CLXXXVI. ANIMAL PHENOLASES AND ADRENALINE

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RECENT work on the enzymic oxidation of adrenaline has suggested that the animal phenolases might play a physiological role in the inactivation of adrenaline liberated at adrenergic nerve endings [Green & Richter, 1937; Bacq, 1938]. Since data are available as to the distribution of the chromaffin system in the invertebrates it appeared that a comparative study of the distribution of the phenolases would help to show if there is any relation between the two systems.

Heard & Raper [1933] showed that adrenaline is oxidized by the catechol oxidase of mealworms and Neuberg [1908] described a phenolase in *Sepia* which inactivates adrenaline. The literature contains a number of other references to the occurrence or absence of phenolases in particular animal tissues [Oppenheimer, 1926; Pugh, 1934] but quantitative data as to the amount of enzyme present are lacking and the enzymes are often ill-defined so that it is not clear to what extent they would oxidize adrenaline. The older workers generally used the colour formation on incubating in the air with an oxidizable phenol such as catechol or quinol as a method of testing for phenolases, but in many cases the *p*H was inadequately controlled so that the enzymic nature of the oxidations described appears doubtful. This method is further unreliable since small amounts of reducing substances such as ascorbic acid or glutathione in the tissue preparations may prevent the appearance of colour.

In the present work the manometric method of measuring the oxygen uptake was used. Preliminary experiments were first carried out with tissue preparations obtained from a series of animals of different phyla in order to obtain an approximate value for the amounts of enzymes of the catechol oxidase type which are present. Homocatechol (3:4-dihydroxytoluene) was used for this purpose in preference to catechol as it is more rapidly oxidized by catechol oxidase and is more closely related to adrenaline and the other naturally occurring catechol derivatives, which generally have a carbon chain attached to the catechol nucleus.

Oxidation of homocatechol by animal tissues

The tissue preparations were made by grinding the tissues for 20 min. with sand, adding 2 vol. of $M/15$ phosphate buffer, centrifuging for 5 min. and making the resulting solution up to a known volume with $M/15$ buffer solution. With tissues such as liver which show a rapid spontaneous O_2 uptake the extract was dialysed and 1/10 vol. $M/1{\cdot}5$ buffer then added before diluting. Buffer of *p*H 7·4 was used except where otherwise stated. With *Aurelia* the extract was buffered with 1/10 vol. $M/1{\cdot}5$ buffer but was not otherwise diluted. The O_2 uptakes were measured by the Warburg method; each cup contained 2 ml. tissue extract and

0·2 ml. water or $M/4$ homocatechol solution. The homocatechol was prepared from vanillin by reduction and demethylation and had B.P. 135°/12 mm. With tissues containing active enzymes the extract was diluted 1 : 6 or 1 : 12 and the O_2 uptake during the first 4 min. was measured. With the relatively inactive tissues the extract was used undiluted and the O_2 uptake was measured during 1 hr. The O_2 uptakes are given in Table I as μl. O_2/g. fresh tissue or blood/hr. Individual specimens often showed considerable variation, but the figures given nevertheless show the order of magnitude of the amount of phenolase present.

With the *Cancer* and *Helix* preparations the oxidation rate showed a slight autocatalytic increase: other systems showed a falling off in the oxidation rate after the first few minutes, but the oxidation rates remained in general approximately constant during the time of measurement. Since the plant phenolases have been more fully investigated than the animal enzymes four plant tissues were included for comparison; experiments showing the effect of adding 10^{-3} M HCN are also given.

In these preliminary experiments no attempt was made to study the properties of the purified enzymes but rather to observe their activity in the presence of the other cell constituents. The purified catechol oxidases are comparatively labile and are rapidly inactivated by the reaction products formed in the oxidation of catechol derivatives [Richter, 1934], but the inactivation is much less rapid when other tissue proteins are present. Many of the extracts showed a red colour during the oxidation, but in some cases the colour was masked by that of the extracts themselves. The small O_2 uptakes, of the order of 100 μl. O_2/g./hr., found for many of the tissues in the presence of homocatechol may be ascribed in part to non-enzymic catalysis by traces of heavy metals and in part to the cytochrome oxidase system which catalyses the slow oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. The large O_2 uptakes, of 3000 or more, observed in several of the arthropods must be attributed to the very active catechol oxidases (also described as "tyrosinases" or "polyphenol oxidases") which are known to occur in animals of this phylum.

In the insect larvae the catechol oxidase is present in the blood but is apparently not confined to the blood since it was found in similar or even higher concentration in the skins which were carefully freed from blood by drying on filter paper, washing with water and pressing on filter paper again. The blood was obtained free from contamination by other tissues by holding the insects firmly between the finger and thumb so as to force the blood towards the middle and then making an incision in the skin.

The vertebrate tissues tested were found to be comparatively inactive, especially in view of the fact that the estimations were done at 37° while 25° was used for most of the other animals. Apart from the arthropods relatively high activity was exhibited only by certain molluscs in which O_2 uptakes of 300–500 μl./g./hr. were obtained. This observation of high activity in the arthropods and molluscs appeared specially suggestive in view of the recent work of Kubowitz [1937] and of Keilin & Mann [1938] who have shown that certain plant phenolases are copper-protein complexes, for it is precisely in the arthropods and molluscs that the copper-containing haemocyanins occur. The systems responsible for the oxidation of catechol derivatives in these two phyla were therefore selected for study in greater detail: the crab *Cancer pagurus* and the edible snail *Helix pomatia* served as convenient sources of material.

Table I. *Rate of oxidation of homocatechol*

Phylum	Species	Tissue	Temp. °C.	μl. O_2/g. tissue/hr. Tissue alone	With substrate	Difference
Coelenterata	*Actinia equina* L.	Whole animal	26	0	69	69
	,,	Whole animal (+HCN)	26	9	61	52
	Tealia felina (L.)	Muscle	24	15	70	55
	,,	Muscle (+HCN)	24	9	25	16
	,,	Mesenterium	26	60	48	−12
	,,	Mesenterium (+HCN)	26	39	32	−7
	Aurelia aurita (L.)	Denser parts	25	0	2	2
Nematoda	*Ascaris lumbricoides* var. *Suis*	Whole animal (*p*H 7·0)	37	51	100	49
Annelida	*Hirudo medicinalis*	Whole animal (*p*H 7·0)	25	4	130	126
	Lumbricus terrestris	Whole animal (*p*H 7·0)	25	0	55	55
Arthropoda	*Odonestis potatoria* (larva)	Blood	25	0	5340	5340
	,, ,,	Skin	25	0	3320	3320
	Lasiocampa quercifolia (larva)	Blood	25	0	623	623
	,, ,,	Skin	25	0	3680	3680
	,, ,,	Viscera	25	64	1065	1001
	Bombyx quercus (larva)	Blood	25	0	3310	3310
	,, ,,	Skin	25	0	8680	8680
	,, ,,	Viscera	25	278	3060	2782
	Eumorpha elpenor (pupa)	Blood	25	0	5130	5130
	,, ,,	Viscera	25	34	3610	3576
	Locusta migratoria (adult: migratory phase)	Viscera	25	494	2440	1946
	Schistocerca gregaria F. (adult: migratory phase)	Viscera	25	192	1105	913
	Dysdercus intermedius Dist. (adult)	Abdomen	25	239	5080	4841
	Periplaneta americana (adult)	Viscera	25	0	141	141
	Cancer pagurus L.	Blood (*p*H 7·0)	30	18	462	444
	,,	Gills (*p*H 7·0)	30	9	256	247
	,,	Hepatopancreas (*p*H 7·0)	30	27	327	300
	,,	Leucocytes (*p*H 7·0)	30	0	4530	4530
	Homarus vulgaris	Blood (*p*H 7·0)	30	7	290	283
Mollusca	*Helix aspersa*	Viscera	25	23	469	446
	Helix pomatia	Blood	30	35	306	271
	,,	Hepatopancreas	30	48	120	72
	Patella vulgata L.	Viscera	24	35	84	49
	,,	Viscera (+HCN)	24	38	75	39
Echinoderma	*Echinus esculentus* L.	Intestine	26	9	56	47
	,,	Intestine (+HCN)	26	7	56	49
	,,	Gonads	26	0	0	0
	Asterias rubens L.	Hepatic caecum	30	7	117	110
	,,	Hepatic caecum (+HCN)	30	5	102	97
Vertebrata	*Rana temporaria*	Liver	37	18	255	237
	,,	Intestine	37	110	107	−3
	Guinea pig	Liver	37	27	197	170
	,,	Intestine	37	7	54	47
	Pig	Kidney	37	72	107	35
	,,	Liver (*p*H 7·0)	37	0	129	129
	,,	Pancreas (*p*H 7·0)	37	6	79	76
	,,	Heart (*p*H 7·0)	37	6	116	110
	,,	Intestine (*p*H 7·0)	37	10	66	56
	,,	Spleen (*p*H 7·0)	37	24	51	27
	Sheep	Liver (*p*H 7·0)	37	11	156	145
	,,	Pancreas (*p*H 7·0)	37	14	87	73
	,,	Kidney (*p*H 7·0)	37	12	194	183
	,,	Heart (*p*H 7·0)	37	6	121	115
	,,	Intestine (*p*H 7·0)	37	6	74	68
	,,	Spleen (*p*H 7·0)	37	0	162	162
	,,	Brain (*p*H 7·0)	37	7	29	23
	,,	Thyroid (*p*H 7·0)	37	0	111	111
	Ox	Liver (*p*H 7·0)	37	17	114	97
	,,	Intestine (*p*H 7·0)	37	30	126	96
Plants	*Agaricus campestris*	Basidium	25	35	>4500	>4465
	Cichorium intybus	Leaf	25	5	>4500	>4495
	Ricinus communis	Seed	25	3	39	36
	Solanum tuberosum	Tuber	25	7	>4500	>4493

Catalytic systems of Cancer pagurus

Haemolymph system. A system that gave rapid colour formation with catechol or homocatechol and O_2 uptakes of 300–800 μl./g./hr. was present in *Cancer* blood or haemolymph. The activity varied considerably from one animal to another. Gills and hepatopancreas were less active than the blood.

	μl./g./hr.		
	Gills	Hepato-pancreas	Blood
Catechol	186	122	795
Homocatechol	153	300	444

Catechol and homocatechol were used for testing the activity of the preparations and the O_2 uptakes were measured at *p*H 7·0 and 30°; otherwise the conditions were the same as are described above. The activity showed a marked increase on dialysis: two specimens of blood gave with homocatechol (*a*) undialysed, 284, 444, and (*b*) dialysed, 445, 1180 μl./g./hr. This is apparently due to an inhibitor which is removed by dialysis. Similar systems were also found in the blood of *Homarus vulgaris*, *Helix aspersa* and the squid *Loligo*, and similar figures were obtained with dialysed and undialysed specimens. The system present in *Cancer* blood had the properties of an enzyme in that it was inactivated by boiling, by digestion with trypsin, by drying or by treating with 95 % alcohol.

Purification. An active preparation was obtained from *Cancer* blood as follows. The animals were cooled to 0° by leaving for a few hours in the ice chest. The blood was obtained by puncturing the heart or severing the dorsal artery and was run directly into centrifuge cups cooled to 0° in ice: each animal gave 50–75 ml. blood. The leucocytes were centrifuged off and the clear liquid was dialysed overnight against distilled water at 0°. The precipitate of protein containing carotenoid pigments which separated was inactive and was centrifuged off. The clear solution containing haemocyanin darkened slowly on keeping but remained unchanged in catalytic activity for several weeks when kept at 0°. Attempts to purify the active system further by precipitation with ammonium sulphate, alcohol or acetone gave rise to gelatinous products which could not readily be centrifuged or filtered.

The catalytic system appeared to be closely associated with the haemocyanin fraction of the blood proteins. Attempts were therefore made to purify the system further by the methods used for crystallizing haemocyanins. To the clear solution obtained by dialysis as described above 5 % acetic acid was added drop by drop with stirring until the solution showed a slight permanent turbidity and gave an orange colour with methyl red (*p*H 4·7). On dialysing again against distilled water at 0° the protein separated out in the form of a colourless crystalline precipitate. The crystals were small but could be seen to be in the form of hexagonal plates when viewed under the microscope.

The crystals slowly turned a pale greenish blue at the surface on exposure to air. They were insoluble in water but dissolved rapidly and completely in dilute salt solutions. A crystalline protein has not previously been obtained from the blood of *Cancer pagurus*. The protein described, while resembling a haemocyanin in its ready solubility in dilute salt solutions, differed from the haemocyanins in being colourless. The aqueous solution showed a very pale greenish colour but did not turn blue or give a measurable O_2 uptake on shaking for 5 min. with air. Copper estimations showed that the protein contained about the right amount of copper for a haemocyanin (Found: Cu, 0·13, 0·12 %). The protein

showed several points of resemblance to the methaemocyanin obtained by Conant *et al.* [1933] by treating haemocyanins with oxidizing agents: it is apparently a modified form of haemocyanin produced during the process of crystallization. The purified crystalline protein showed considerable catalytic activity when tested in the usual way with homocatechol, the activity corresponding to that originally present in the blood: the mother liquor from which the crystals were obtained showed practically no activity. The activity also remained constant on recrystallizing.

Table II. *Catalysis by crystalline copper-protein complex*

	Dialysed blood (33·3 mg./2 ml.)		Crystalline prep. (28·6 mg./2 ml.)	
	μl. O_2/hr.	Q_{O_2}	μl. O_2/hr.	Q_{O_2}
Catechol	415	12·5	466	16·3
Homocatechol	170	5·1	239	8·3
Adrenaline	4	0·1	12	0·4

Although the purified *Cancer* preparation was very active when tested with catechol and homocatechol it was found, surprisingly, that it showed comparatively little activity with adrenaline as substrate. The catalytic activity was strongly inhibited by cyanide and also by the copper reagent cupferron. The inhibitions found at a concentration of 1/2000 inhibitor in the oxidation of catechol under the conditions previously described were: HCN, 95 %; NaF, 43 %; cupferron, 69 %; sodium diethyldithiocarbamate, 9 %; Na_2S, 24 %; 8-hydroxyquinoline, 29 %. The activity of the preparation was not appreciably changed by treating with dilute acetic acid (*p*H 3·0) for 4 hr. Under these conditions the haemocyanin is known to undergo modification so that the copper, although it is still combined and cannot be removed by dialysis, becomes capable of catalysing the oxidation of thiol derivatives [Pirie, 1931]. In the crystalline preparation the dialysis at *p*H 4·7 may have already brought about this modification.

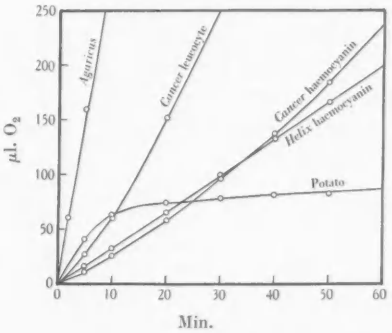

Fig. 1. Oxidation of homocatechol. Conditions as given for Table I. Protein content of preparations in 2·2 ml.: *Agaricus*, 1·7 mg.; *Cancer* leucocyte, 5·9 mg.; *Cancer* haemocyanin, 28·6 mg.; *Helix pomatia* crystalline haemocyanin, 12·0; potato, 0·6 mg.

Cancer *leucocyte catechol oxidase*. Experiments with crude extracts of gills and hepatopancreas showed that these extracts, unlike the purified preparations from centrifuged blood, catalysed the oxidation of adrenaline. This indicated

the presence of a second catalytic system, which was found in highest concentration in the leucocytes. Pinhey [1930] concluded from colorimetric experiments that crustacean bloods contain a phenolase which is liberated by the bursting of the "explosive corpuscles" of Hardy. In the present investigation Pinhey's observations were confirmed.

The leucocytes were separated from the blood by centrifuging at 0°, washing rapidly with saline and centrifuging again. The extract prepared by grinding with sand in the usual manner contained a very powerful phenolase which catalysed the oxidation of adrenaline as well as that of catechol and homocatechol; the extract also catalysed the oxidation of monohydric phenols such as *p*-cresol.

	μl. O_2/g. tissue/hr.	Q_{O_2} of preparation (μl./mg. dry protein/hr.)
Catechol	2820	49
Homocatechol	4830	83
Adrenaline	5840	100

The Q_{O_2} values obtained for the leucocyte preparations were much higher than with the blood plasma preparations. The enzyme present was similar in activity and specificity to the well-known mealworm catechol oxidase.

Catalytic system of Helix pomatia

In the snail, as in the crab and lobster, the blood contains a system that gives colour formation and O_2 uptake with catechol or homocatechol. Here again the system was closely associated with the haemocyanin fraction of the blood proteins. Crystalline haemocyanin prepared by the method of Dhéré [1919] showed a considerable catalytic activity corresponding to that of the blood. The system was relatively inactive with adrenaline as substrate.

	Dialysed *Helix* blood (14·6 mg. protein in 2 ml.)		Crystalline haemocyanin (12 mg. protein in 2 ml.)	
	μl. O_2/hr.	Q_{O_2}	μl. O_2/hr.	Q_{O_2}
Catechol	160	10·9	134	11·2
Homocatechol	196	13·4	176	14·6
Adrenaline	25	1·7	30	2·5

Catalysis by heavy metals

That traces of heavy metals can catalyse the oxidation of phenols is well known (a good review of the literature is given by Sutter [1936]). In view of their similarity to the plant enzymes the animal catechol oxidases are probably also Cu-protein complexes. The observed catalytic activity of the haemocyanin preparations raises the question of how far the activity of the other phenolases described in the literature may be ascribed to the specific or unspecific catalysis by heavy metals or heavy metal-protein complexes. The activity of the haemocyanin preparations might be attributed to traces of loosely combined Cu present as an impurity in the preparations. It therefore appeared desirable to determine the catalytic activity of traces of heavy metals under the conditions used throughout and to check this against the actual heavy metal content of the preparations.

For several of the substrates tested there was a large increase in the oxidation rate as shown by the colour formation and O_2 uptake on adding Cu, Ni or Co at concentrations of the order of 0·1 mg. metal/ml. Of the commoner heavy metals tested Fe was comparatively inactive as a catalyst; Cu was the most

Table III

O_2 uptakes (μl./hr.) in the presence of heavy metals at *p*H 7·0 and 30°. Each Warburg vessel contained 0·2 mg. metal in 2 ml. *M*/15 phosphate buffer and 0·2 ml. *M*/4 substrate. The metals were added in the form of the sulphates.

Substrate	No addition	Cu	Fe	Ni	Co	Mn
Catechol	2	45	12	119	65	13
Homocatechol	34	238	59	313	163	59
Adrenaline	6	115	55	266	251	2
Guaiacol	0	0	0	2	4	0
Protocatechuic acid	2	0	0	8	19	0
Pyrogallol	82	115	42	286	218	76
Phloroglucinol	0	3	0	3	16	3
p-Cresol	0	0	0	0	6	0
Quinol	4	18	2	8	12	20
p-Phenylenediamine	0	198	0	0	135	0

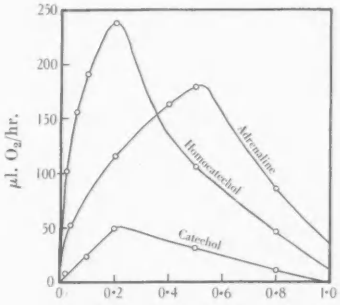

Fig. 2. Effect of Cu concentration on the rate of oxidation. The manometers contained 2 ml. *M*/15 phosphate buffer of *p*H 7·0 and 0·2 ml. *M*/4 substrate. Experiments at 30°.

active of those present in significant amounts in most animal tissues. Using Cu as a catalyst the rate of oxidation increased with the Cu concentration (Fig. 2) until high concentrations were reached at which the amount of Cu present was comparable with the amount of phenol, when there was a falling off in oxidation rate. The effect of adding gelatin to the system to form the copper-gelatin complex depended on the substrate and on the Cu concentration, but no very large increase in catalytic activity was observed. The gelatin used was purified by the method of Northrop & Kunitz [1927].

Table IV

O_2 uptakes (μl./hr.) at different Cu concentrations, with and without gelatin. Conditions as in Table III. The figures given for the Cu concentration represent mg. Cu in each Warburg vessel (total vol. 2·2 ml.). The series marked "Gel." contained 7 mg. gelatin.

	Cu concentration											
	0·02		0·05		0·1		0·2		0·5		1·0	
Substrate	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.
Homocatechol	102	76	149	100	193	135	238	190	108	177	15	109
Catechol	10	25	14	31	20	38	60	63	34	71	0	38
Adrenaline	46	115	—	—	—	—	115	190	182	225	37	154
Pyrogallol	92	92	—	—	—	—	115	116	133	165	126	220

Although the catalytic activity of Cu is very considerable these experiments show that the activities of the haemocyanin and leucocyte systems are much greater than that of an equivalent amount of inorganic Cu: their activities cannot therefore be due to small amounts of Cu present as an impurity in the preparations. Inorganic Cu is also much less specific in that it catalyses the oxidation of *p*-phenylenediamine, for example, which is not oxidized by the haemocyanin system or the catechol oxidases. This is shown in Table V in which the specificities of a number of phenolases and *pseudo*phenolases are compared.

Table V. *Specificity of catalytic systems*

O_2 uptakes (μl. O_2/2 ml./hr.) at *p*H 7·0 and 30°. Conditions as for Table I.

	Cancer haemocyanin	*Helix* haemocyanin	*Cancer* leucocyte	Potato	*Agaricus*	*Dolichos*	Cu	Buffer only
mg. protein/2 ml.	27·3	14·6	5·9	0·6	0·3	3·3	0	0
γ-Copper/2 ml.	32·8	32·2	6·5	0·5	0·4	0·3	200	<0·1
Catechol	466	160	286	176	393	229	45	2
Homocatechol	189	196	490	415	385	139	238	34
Adrenaline	21	25	591	210	365	334	115	6
Protocatechuic acid	0	2	3	16	1	5	0	2
Guaiacol	5	0	5	—	1	—	0	0
p-Cresol	2	9	742	248	189	0	0	0
Phloroglucinol	2	5	0	72	1	3	3	0
Quinol	12	19	12	36	61	20	18	4
Pyrogallol	231	122	260	185	263	76	115	82
p-Phenylenediamine	12	7	0	9	1	10	198	0

The enzymes were prepared from the mushroom *Agaricus campestris* (basidium), the legume *Dolichos* (seed) and potato tuber by grinding the tissue with sand, extracting with buffer of *p*H 7·0, precipitating with alcohol and dialysing. The Cu estimations which are included in Table V were done colorimetrically with sodium diethyldithiocarbamate.

The large differences in the specificity of the systems towards different substrates are very striking, and are sufficiently well-defined to be used for identifying them. The *Cancer* leucocyte enzyme shows a specificity similar to those of the mushroom and potato enzymes. Inorganic Cu differs both from the phenolases and from the haemocyanin systems in specificity, but in catalysing the oxidation of *p*-phenylenediamine it resembles the cytochrome system: on other grounds there is evidence that the cytochrome oxidase is a Cu-protein complex [Keilin & Hartree, 1938, 2].

Discussion

(*a*) *Catalytic activity of haemocyanins.* Catechol and homocatechol gave rapid O_2 uptakes accompanied by colour formation when shaken with the blood of species of *Cancer*, *Homarus*, *Loligo* and *Helix*. The natural inference was that this was due to phenolases in the blood, and this appeared to be confirmed when the systems were found to be thermolabile and to show other enzymic properties. Attempts to purify the phenolases then led to the preparation of purified specimens of haemocyanin or Cu-protein complexes derived from the haemocyanins, one of which was obtained crystalline. Crystalline *Helix* haemocyanin also showed considerable phenolase activity. It appeared possible that the catalytic activity might be due to traces of very active phenolase present as an impurity, but this became improbable when it was found that the activity of the haemocyanins remained constant on recrystallization and was higher than that of the unpurified blood proteins. It could be shown that the catalytic activity of the haemocyanins was not due to inorganic Cu contained in the preparations

since an equivalent amount of Cu was much less active and less specific as a catalyst than when combined in the form of haemocyanin. It is therefore concluded that the haemocyanins are themselves catalytically active and can act as *pseudo*phenolases. This *pseudo*phenolase activity of the haemocyanins is comparable with the *pseudo*peroxidase activity of the haemoglobins.

Experiments on the mechanism of the catalysis indicated that a modified form of haemocyanin, possibly methaemocyanin formed during the experiment by the action of traces of *ortho*quinones, is more active than haemocyanin itself since (*a*) the oxidation curves were autocatalytic in shape, (*b*) the modified form of haemocyanin produced by treatment with acid was equally active and (*c*) adrenaline, the *ortho*quinone of which is very labile, was not appreciably oxidized.

The catalytic activity of the haemocyanins is much lower than that of the catechol oxidases, but it is sufficient to give a strongly positive phenolase test by the colorimetric method and to account for the apparent phenolase activities of a number of arthropod and mollusc tissues.

(*b*) *Distribution of phenolases.* Catechol oxidases are present in a number of arthropods, including the crab *Cancer pagurus* where the enzyme occurs chiefly in the leucocytes. Active phenolases of this type were not found in animals of any other phylum. The number of animals tested is small, but in those hitherto tested apart from the arthropods the results show that if any catechol oxidase is present the amount must be comparatively small.

The literature contains many references to the existence of phenolases in other animal phyla [Oppenheimer, 1926], but the colorimetric technique has generally been used for showing their presence. By the colorimetric method the general practice has been to incubate the tissue extracts at alkaline reaction and 37° with an oxidizable phenol such as "dopa" and examine the extracts after 12–24 hr. for colour formation [Bloch, 1917; Pugh, 1933]. Colour formation under these conditions is an extremely sensitive test which may be given by a number of different types of catalysts. A phenolase which is so inactive that it can be detected only in this way must have quite a different order of activity from the arthropod or plant enzymes, which give high Q_{O_2} values and a coloration with homocatechol at *p*H 7·0 and 25° in less than a minute.

The present observations on the *pseudo*phenolase activity of Cu-protein complexes show that colour formation in the presence of an oxidizable phenol, even when supported by evidence of thermolability, cannot be taken as sufficient evidence of the existence of a phenolase. Thermolability is unreliable as a criterion of the enzymic nature of a phenolase as small amounts of heavy metals may remain adsorbed on the protein and so may be removed from solution when a protein is coagulated: Stotz *et al.* [1937] showed that artificially prepared Cu-protein complexes resemble enzymes in their thermolability. Cu is present in many animal tissues in sufficient amounts to catalyse the slow oxidation of catechol derivatives; Cunningham [1931] found as much as 0·1–0·9 mg. Cu/g. in a number of animal tissues while 0·025 mg. Cu/g. is sufficient to give a positive colorimetric "phenolase" test with "dopa".

It has recently been shown that the cytochrome oxidase system which is present in many animal tissues can also catalyse the oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. In view of these observations the enzymic nature of a number of the phenolases described in the literature would appear to be doubtful.

(*c*) *Physiological function of phenolases.* The available evidence gives no support to the view that the catechol oxidases are in any way related to the

chromaffin system in their function. The highest phenolase activity was found in the arthropods, in which no chromaffin system has been found, while *Lumbricus* and *Hirudo* which have well-defined chromaffin systems showed little phenolase activity.

There seems no reason to believe that the *pseudo*phenolase activity of the haemocyanins is of any physiological significance. The very active phenolase of *Cancer* leucocytes, on the other hand, may be expected to come into action by the disintegration of the corpuscles when bleeding owing to an injury occurs [Pinhey, 1930]: the rapid darkening of the shed blood gives evidence of the activity of the liberated phenolase and this is seen particularly clearly with *Maia squinado* and many insects in which the blood rapidly turns nearly black. The physiological function of the phenolases may perhaps be sought in the properties of the *ortho*quinones to which they give rise, such as their activity as respiratory carriers or their bactericidal action.

Summary

1. A number of animal tissues have been examined for the presence of phenolases of the catechol oxidase type.

2. A crystalline Cu-protein complex which is catalytically active has been obtained from the blood of *Cancer pagurus*.

3. Haemocyanins and other Cu-protein complexes can act as *pseudo*phenolases: they are mainly responsible for the apparent phenolase activity in a number of arthropods and molluscs.

4. The catalytic activities of Cu, Fe, Co, Ni and Mn in the oxidation of a series of phenols have been examined.

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CLXXXVII. THE DISTRIBUTION OF THE SUCCINIC OXIDASE SYSTEM IN ANIMAL TISSUES

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In recent work two functions have been suggested for the enzyme system which catalyses the oxidation of succinate to fumarate in many animal tissues. In muscle and in kidney the oxidation of succinate to fumarate appears to be a step in a cycle of reactions involved in the oxidative breakdown of lactate and pyruvate or of triose [Toenniessen & Brinkman, 1930; Elliott & Schroeder, 1934; Elliott *et al.* 1935; Elliott & Greig, 1937; Krebs, 1937]. According to Szent-Györgyi and his collaborators [e.g. Szent-Györgyi, 1935, 1937] the succinate-fumarate system occupies a central position in the system of oxidation-reduction catalysts which are responsible for tissue respiration, i.e. for the oxidation of cell metabolites in general. The enzymes concerned in succinate oxidation are known to be widely distributed and to occur in high activity in some tissues [Battelli & Stern, 1910]. However, in two types of tumour tissue which respired at a rate comparable with that of normal tissues, Elliott *et al.* [1935] found practically no succinate-oxidizing activity. It therefore seemed that useful information concerning the mechanism of tissue respiration would be obtained by a quantitative survey of the succinate-oxidizing activity and of the enzymic components responsible for succinate oxidation in various types of tissue. The results of such a survey are here presented.

It is generally accepted [Szent-Györgyi, 1924; Fleisch, 1924; Keilin, 1929] that the oxidation of succinate occurs under the influence of two enzymes and a "carrier". One of the enzymes, the dehydrogenase, is specific for succinate and under its influence succinate can be oxidized to fumarate while methylene blue, for instance, accepts H atoms and is reduced to leucomethylene blue. In ordinary circumstances, without methylene blue, the H acceptor is oxidized cytochrome. The cytochrome thus reduced acts as a H carrier and is reoxidized by molecular O_2 under the influence of the long known "indophenol oxidase" which Keilin [1929] has identified with the "respiratory ferment" of Warburg. Following the usual terminology for enzymes, this enzyme is best described as "cytochrome oxidase" since cytochrome is the only known substrate for it. *p*-Phenylene diamine and the "Nadi reagent" (dimethyl-*p*-phenylenediamine + α-naphthol) have been mentioned as substrates for this enzyme, but it seems clear (see below) that these substances also are oxidized through the mediation of cytochrome.

For the purpose of this paper the following terms will be used:

"Succinoxidase" will mean the entire aerobic succinate-oxidizing complex of oxidase, cytochrome and dehydrogenase.

"*p*-Phenylenediamine oxidase" will mean the *p*-phenylenediamine-oxidizing complex of oxidase and cytochrome.

"The oxidase" will mean cytochrome oxidase.

"The dehydrogenase" will mean succinic dehydrogenase. It seems certain that no separate coenzyme is involved in this dehydrogenase activity [Boyland & Boyland, 1934; Andersson, 1934].

Rosenthal [1937] has suggested that the determination of the rate of succinate oxidation in very thin slices of tissue could be used as a means of obtaining a minimum estimate of the respiratory ferment in intact tissue. While the determination of the activity of the succinoxidase in intact cells can best be determined in this way, it will be shown below that the full activity of the respiratory ferment is not displayed unless there is excess of the dehydrogenase and cytochrome present in the cells. Estimates of the full respiratory ferment activity of various tissues have been made by determination of the rate of *p*-phenylenediamine oxidation by broken up tissues in the presence of excess cytochrome.

Experimental

General methods

Except in a few specified experiments the tissues studied were prepared by "homogenization" of known weights of tissue in definite volumes of *M*/10 phosphate buffer, *p*H 7·4. The apparatus recently described by Potter & Elvehjem [1936], which reduces tissues rapidly and conveniently to a fine suspension which can be pipetted, was used. In most cases the tissue was chopped up with scissors whilst muscle was passed through a Latapie mincer, before homogenization. With some rather tough tissues the homogenization took several minutes and the homogenizer tube was kept cool in ice water. It was sometimes difficult to break up the last few pieces of tissue without continuing the homogenization for a long time; the weight of these pieces was determined and deducted from the total amount taken. Tissues from freshly killed animals were used; the suspension was kept in a refrigerator between experiments and it was used only during the day on which it was prepared. The suspensions contained amounts of moist tissue ranging from 50 mg. (heart, liver, kidney) to 200 mg. (spleen, tumours) per ml. With many tissues it was necessary to take material from a number of rats to obtain enough suspension for a set of experiments. In every experiment samples of the chopped tissue before homogenizing were weighed and dried at 100–110°, to determine the wet weight/dry weight ratio.

For the determination of O_2 uptake rates, simple Barcroft differential manometers were used [see Dixon, 1934]. The vessels contained air and a total of 3 ml. fluid, of which 1 ml. was *M*/10 phosphate buffer, *p*H 7·4, added separately or with tissue suspension. Homogenized tissue suspensions were introduced by pipette; in experiments with minced or chopped tissue this was weighed in the dry vessel before adding the solutions. The taps were closed 6 min. after immersing the vessels in the bath at 38° and readings were taken every 5 min. for 25 min. The rate of shaking was 132 oscillations/min. and it was found that O_2 uptakes of well over 1000 μl./hr. could be measured without uncertainty due to diffusion effects. In general, amounts (0·25–2 ml.) of tissue suspension were chosen such that the O_2 uptake was well below 1000 μl./hr. In the large majority of experiments the O_2 uptake/time curve was linear, at least after the first 5 min. The rate was taken from the linear part of the curve or in the few cases where the rate fell off appreciably the initial rate was accepted.

Succinoxidase

For the estimation of succinoxidase activity, equal amounts of the suspension were pipetted into both vessels of the Barcroft manometer and neutralized succinic acid solution was measured into the right-hand vessel. No alkali papers were put in the inner cups since no CO_2 is evolved in the oxidation of succinate to fumarate. Elliott & Schroeder [1934] showed that kidney slices oxidized fumarate rapidly, while with finely minced kidney practically no oxidation with fumarate took place though the succinic oxidation still occurred rapidly. Experiments with homogenized kidney, muscle, and other tissues, showed that no appreciable oxidation of fumarate occurred with the amounts of tissue used and at the concentration of fumarate which was likely to be present.[1]

In order to obtain a comparison of the succinoxidase activities of various tissues, it was first necessary to determine the conditions under which maximum activity would be observed in any tissue. While disintegration of tissue does not destroy succinoxidase, it is known [e.g. Lehmann, 1929] that violent shaking does inactivate it. With tissues having high activity, experiments showed that the activity observed after homogenization by Elvehjem's apparatus was greater than with minced or chopped tissue, probably owing to better dispersion. With several tissues having low activity, homogenizing did decrease the activity somewhat; it is possible that the violent breaking up of these tissues which were all rather tough has some destructive effect on the enzymes appreciable in comparison with the small activity of these tissues (see Table I).

Potter & Elvehjem [1937] carried out some measurements of succinoxidase activity, but the results given by them are considerably lower than those reported below (e.g. for rat kidney and liver suspension they found O_2 uptakes respectively of 5932 and 3895 μl./hr. per 1000 mg. tissue, against our figures of 22,600–33,200 and 13,300–24,000). Possibly their method of using only 1 ml. of fluid instead of 3 ml., in order to facilitate diffusion, was not quite satisfactory with the high concentration of tissue (40 mg.) which they used. They found that the maximum O_2 uptake occurred with 0·3 % succinic acid in the medium. In this work with all the tissues studied the optimum concentration was found to be about 0·9 %, and this concentration was in general used, though in most cases the O_2 uptake was measured at two or more different concentrations. The rate was often appreciably smaller with 0·6 % or less, or with 1·2 % succinic acid.

It is known that breaking up a tissue into a suspension lowers the respiration considerably compared with the respiration of thin slices. Elliott & Schroeder [1934] suggested that this is due to a dilution of coenzymes etc. and Krebs [1935] and Potter & Elvehjem [1936] showed that with a suspension the rate of respiration per unit weight increases with increasing concentration of tissue.[2] However, Potter and Elvehjem [1936] found that, unlike the residual respiration, the succinoxidase activity of tissues per unit weight is independent of the concentration of the tissue. We have confirmed this with all the tissues studied, by varying the amount of tissue in the 3 ml. of medium; also, in two experiments, the same rate of O_2 uptake was observed with equal small amounts of liver

[1] In later experiments it was found that fumarate increased the O_2 uptakes when higher concentrations of tissue were used. The extra O_2 uptake was largely balanced by extra CO_2 evolution.

[2] With the very dilute suspensions in phosphate buffer solution used in this work, the O_2 uptake in the absence of added substrate varied between 0 and 20 μl. in the 25 min. experimental period.

tissue in 3 ml. or in only 1 ml. of medium. Nevertheless, the succinoxidase activity was determined with two or more concentrations of tissue in nearly every case.

It was found that the activity of a liver, kidney, or heart tissue suspension sometimes, but not always, increased up to 35% (70% in one instance) during 1 or 2 hr. standing in the refrigerator. After this period the activity remained constant for a long time. Presumably slow solution of constituents of the tissue results in the better dispersion of some constituent of the enzyme system. In nearly every case the succinoxidase activity was determined at least twice,

Table I. *Succinoxidase activities of rat tissues*

Tissue	Preparation	Succ Q_{O_2} On moist wt.	Mean of homogenized On dry wt.
Whole kidney	Homogenized	33·2, 30·4, 22·6, 18·9	112
Liver	Latapie minced	13·9	—
	Homogenized	20·2 | 24·0, 13·3, 25·8, 21·8, 16·1, 13·8	66
Heart	Chopped	7·3	—
	Homogenized	18·4 | 12·8, 12·9, 13·0, 12·1, 13·2	62
Whole brain	Ground to paste	3·9	—
	Homogenized	3·8 | 4·2, 3·7, 3·2, 3·5, 4·0, 4·6, 4·3, 6·2	18
Testis	Ground to paste	1·6	—
	Homogenized	1·9 | 1·5, 1·2, 1·7, 1·1, 1·9, 2·0, 1·7	13
Adrenal	Homogenized	3·6	11·7
Lung*	Chopped	0·6	—
	Homogenized	1·8 | 1·8, 0·9	7·5
Skeletal muscle	Latapie minced	2·5 | 3·1 | 2·4	—
	Chopped	2·3	—
	Homogenized	1·5 | 1·7 | 1·4 | 1·9, 1·3	6·6
Thymus	Chopped	0·51	—
	Homogenized	0·33	1·6
Pancreas	Chopped	0·24 | 0·13	—
	Homogenized	0·0 | 0·02	0
Spleen	Chopped	0·13	—
	Homogenized	0·16 | 0·23, 0·04, 0·09	0·5
Blood†	Laked	0, 0	0
Whole foetus (1·1 g. each)	Chopped	0·73	—
	Homogenized	1·90	17·4
Jensen sarcoma (intramuscular)	Chopped	0·6‡	—
	Homogenized	2·1 | 2·7, 2·1	13
Jensen sarcoma (subcutaneous)	Homogenized	2·0	12·9
Flexner Jobling carcinoma	Homogenized	2·0, 0·4	12·8, 2·1
Walker No. 256 carcinoma	Chopped	0·08	—
	Homogenized	0 | 0·1, 0·1, 0·16, 1·0	0·6, 7·2
Philadelphia No. 1 sarcoma	Chopped	0·13	—
	Homogenized	0·11 | 0·40, 0	1·0
Spontaneous mammary carcinomata	Chopped	0·21	—
	Homogenized	0·03 | 0·18	0·5

* The O_2 uptake rates of rat and rabbit lungs with succinate fell off rapidly with time.

† The blood was taken from the hearts of etherized animals and run into water containing a trace of heparin. Tests showed that neither traces of ether nor of heparin affected succinoxidase.

‡ Tissue clumped together and rate fell off rapidly.

Table II. *Succinoxidase activities of rabbit and other tissues*

Tissue	Preparation	Succ Q_{O_2} On dry wt.
Rabbit kidney cortex	Homogenized	65·7
Rabbit kidney medulla	Homogenized	24·5
Rabbit liver	Latapie minced	34·5
	Homogenized	36·2
Rabbit heart	Homogenized	150
Rabbit testis	Homogenized	9·2
Rabbit lung	Chopped	6·1
	Homogenized	8·3
Rabbit skeletal muscle	Latapie minced	2·1
	Homogenized	1·5
Chick embryo (7 day)	Chopped	0·7
	Homogenized	2·0
Ox retina	Intact	10·3 } 3·9 }
	Homogenized	10·1 } 11·9 }

at intervals of 1 or 2 hr. When this was not convenient the determination was made 2 or 3 hr. after preparing the suspension.

To express succinoxidase activity the term Succ Q_{O_2} will be used where

$$\text{Succ } Q_{O_2} = \frac{\mu\text{l. } O_2 \text{ taken up in the oxidation of succinate}}{\text{hr.} \times \text{mg. tissue}}.$$

Tables I and II give the activities found in a number of tissues. The results are calculated on the moist weight and on the dry weight of tissue taken, the dry weight being deduced from the wet weight/dry weight ratio of the separately dried sample of tissue.

It will be seen in Tables I and II that the distribution of activity is very similar in the tissues of the rabbit and the rat. According to their succinoxidase activities the tissues fall roughly into three groups. (1) Kidney, liver and heart muscle have very high activities.[1] (2) Brain, testis, skeletal muscle, lung, adrenal, retina, Jensen sarcoma and Flexner-Jobling carcinoma, have fairly low activities. (3) Thymus, pancreas, spleen, blood, Philadelphia No. 1 sarcoma (usually), Walker No. 256 carcinoma, and two spontaneous mammary carcinomata (rat) have only a trace of activity or none at all. Battelli & Stern [1910] noted the high activities of kidney, liver and heart, but the activities observed with the crude methods available to them were several times lower than those now found. At present there does not seem to be any clear connexion between metabolic activity and succinoxidase activity. It is interesting to find tissues with normal respiratory activity but with practically no succinoxidase, as with spleen and several tumours. Absence of succinoxidase is not a definite characteristic of cancer tissue since some types of tumours, e.g. the Jensen sarcoma, always have definite activity. Fleisch [1924] and Boyland & Boyland [1936] have also found succinoxidase in the Jensen sarcoma. It does not seem to be a characteristic even of a single strain since with the Walker No. 256 one crop[2] was found to be quite active.

[1] The activity of these tissues is so high that very dilute suspensions of them are useful as quickly obtainable preparations of "succinoxidase" for use in rough estimations of succinate.

[2] For a set of experiments with small tumours, five or six young tumours implanted at the same time in several rats from one tumour were used.

The dehydrogenase

The succinoxidase activity as measured in the previous section is liable to be limited by the amount of any one of the components of the catalytic system. It was of interest, therefore, to obtain separate estimates of the specific succinate enzyme, i.e. the dehydrogenase, by an adaptation of the well-known methylene blue reduction technique of Thunberg. In the reduction of methylene blue no O_2 activator or carrier is concerned and only the rate of H transfer from succinate to methylene blue under the influence of the dehydrogenase is measured.

The Thunberg tubes used were of the Keilin [1929] type with a bulb in the stopper. The stopper bulbs contained 0·5 ml. 0·703 % methylene blue, this concentration being chosen so that 0·475 ml. contains methylene blue equivalent as H acceptor to 10 μl. O_2. The main tube contained a total of 2·5 ml. fluid consisting of tissue suspension in $M/10$ buffer, neutral succinate solution, water and enough extra buffer to make with the tissue suspension a total of 1 ml. phosphate buffer ($M/10$, pH 7·4). Control tubes contained the same additions with water instead of succinate solutions. Up to 10 tubes were evacuated simultaneously by means of a brass tube with 10 side tubes, connected to an oil suction pump and manometer. The tubes were evacuated three times and refilled with N_2 freed from O_2 by passage through a tube of hot copper wire fragments. They were finally evacuated, firmly closed, and the side tubes filled with water. In order to prevent the tissue from settling out, the tubes were fixed into a series of clips on a horizontal beam which was attached to the shaking apparatus of an ordinary Barcroft manometer tank; the tubes used were bent through an angle of about 40° and the clips were fixed at such an angle that the main parts of the tubes were nearly horizontal and the motion of the shaker kept the contents of the tubes well mixed. The main parts of the tubes were immersed in the bath water at 38°. The tubes were shaken in the bath for 1–3 min. before mixing the methylene blue at zero time. 1 min. was found to be sufficient to bring the fluid almost to the temperature of the bath. In open test tubes, mixtures were made of the same amounts of tissue suspension and buffer as in the Thunberg tubes, with water added to make a total of 3 ml., and 0·025 ml. of the methylene blue solution were added. These comparison tubes were frequently shaken with air to keep the methylene blue oxidized. The time of reduction was taken as the time when the colour in the Thunberg tube matched the colour in the comparison tube containing the same amount of tissue.

The activity of the succinic dehydrogenase will be expressed by the term Succ Q_{MB} where

$$\text{Succ } Q_{MB} = \frac{\mu\text{l. } O_2 \text{ equivalent of methylene blue reduced by the succinate system}}{\text{hr.} \times \text{mg. tissue}}$$

$$= M\frac{t - t_{Succ}}{t} \times \frac{60}{t_{Succ} \times w},$$

where $M = O_2$ equivalent of the total methylene blue reduced,

t = time in min. for reduction in the absence of succinate,

t_{Succ} = time in min. for reduction in the presence of succinate.

$\frac{t - t_{Succ}}{t}$ = fraction of total methylene blue reduced which is reduced by succinate,

w = weight of tissue in mg.

Table III. *Examples of the determination of succinic dehydrogenase activity of homogenized rat tissues*

Tissue	mg. moist	t	t_{Succ}	Succ Q_{MB}	Tissue	mg. moist	t	t_{Succ}	Succ Q_{MB}
Kidney	20	∞	8, 8, 8	3·8	Muscle	200	23, 28	8·5, 8·5, 8·5	0·24*
	10	∞	15, 15	4·0		100	77, 84	14·5, 14·5, 14·5	0·34
	5	∞	38, 38, 43	3·0		50	200±	24, 21·5, 22	0·47
		6 hr. later					4 hr. later		
	20	∞	8, 8, 8	3·8		100	72, 75	14, 14·5	0·34
	10	∞	13, 16, 16	4·0		50	200±	23, 23	0·46
	5	∞	32, 34, 34	3·7		25	500±	51, 51, 49	0·46
Liver	50	109	6, 5·5	2·0	Spleen	200	10·5, 10·5	7, 7	0·14*
	25	360±	12, 12	1·9		100	32, 32	12, 12	0·31
	12·5	∞	23, 26	2·0		50	155±	29, 28	0·34
		3 hr. later							
	12·5	∞	27, 27	1·8	Blood	240	∞	∞	0
						120	∞	∞	0
Heart	50	210±	3·5, 3·5	3·4*		60	∞	∞	0
	25	350±	6, 6	3·9	Jensen sarcoma	133	50, 40	15, 15, 16	0·20
	12·5	∞	10·5, 10·5	4·6		70	120, 120	38, 31	0·18
	12·5	∞	12, 11·5	4·1		35	∞	105, 89	0·12†
	6·25	∞	25, 26	3·8					
	3·13	∞	67, 67	2·9†	Walker No. 256 carcinoma	195	40, 48	19·5, 14·5	0·11
Brain	100	35, 40	5·5, 5·5	0·95		98	190±	49, 59	0·08
	50	126, 141	12·5, 13	0·86					
	25	600±	26, 26	0·88	Phila. No. 1 sarcoma	200	7·5, 6·5	5·5, 5, 5·5	0·15*
Testis	200	23, 21·5	8·5, 8·5	0·22		160	25, 29	8, 8	0·33
	100	60, 61	20, 19	0·20		100	86, 74	22·5, 22·5	0·19
	50	425±	43, 39	0·26		80	108±	46, 46	0·09†
Lung	200	27, 35	7, 7	0·37					
	100	171±	34, 34	0·16†					
	50	∞	83, 83	0·16†					

Accurate determination of the reduction time with spleen is difficult due to the large amounts of haemoglobin present which is reduced in the vacuum tubes and oxygenated in the comparison tubes.

* The method often does not show the maximum activity when the reduction time is very short.

† With long reduction times inactivation of the enzyme seems to occur. With lung this is particularly marked. The succinoxidase activity of lung (rat and rabbit) also falls off rapidly in aerobic experiments.

Table IV. *Succinic dehydrogenase activities of homogenized rat tissues*

Tissue	Succ Q_{MB}*		Tissue	Succ Q_{MB}*	
	On moist wt.	Mean on dry wt.		On moist wt.	Mean on dry wt.
Kidney	3·9, 2·2	12·9	Muscle	0·42, 0·51, 0·59	2·1
Liver	2·0, 1·3, 1·8, 1·4	5·1	Spleen	0·25, 0·31, 0·32	1·3
Heart	3·4, 4·1	17·1	Blood	0	0
Brain	0·81, 0·88	3·9	Jensen sarcoma	0·20, 0·22	1·3
Testis	0·16, 0·23	1·5	Walker No. 256 carcinoma	0·06, 0·10	0·5
Lung†	0·32, 0·38	1·8	Phila. No. 1 sarcoma	0·22	1·3

* Widely variant results, see footnotes to Table VII, omitted.

† Uncertain, see footnote to Table III.

Under the conditions chosen for these experiments, $M = 10$,

whence $$\text{Succ } Q_{MB} = \frac{600}{t_{Succ} \times w} \times \frac{t - t_{Succ}}{t},$$

or when the reduction by the tissue alone is very slow compared with the time in the presence of succinate, i.e. when t is much greater than t_{Succ},

$$\text{Succ } Q_{MB} = \frac{600}{t_{Succ} \times w}.$$

This formula is of course only an approximation, since it is assumed that the rate of reduction by substrates in the suspension is unchanged by the presence of succinate, and that the rate of reduction by succinate and other substrates is independent of the methylene blue concentration. Further uncertainty may be introduced by adsorption of the dye on tissue particles, and by progressive inactivation of both the succinic dehydrogenase and the other reducing mechanisms. However, the method serves to give a rough comparison of the dehydrogenase activities of the various tissues. While the time of reduction without succinate (t) tends to increase very rapidly with dilution of the tissue, the succinic dehydrogenase activity as measured by this method is usually approximately proportional to the amount of tissue. Occasionally rather wide variations in Succ Q_{MB} were found, especially with tissues of low activity, or when the reduction time was very short or very long. No appreciable change in activity was noticed in suspensions kept in the ice box for a few hours. Table III illustrates these points. The optimal concentration of succinate was found to be about 0·2–0·5 %; with very low concentrations the maximum rate is not reached, and higher concentrations cause definite inhibition. It will be noticed that the optimal concentration of succinate is lower for the reduction of methylene blue than for O_2. It is possible that excess succinate causes over-saturation of the enzyme surface preventing access of the dye, while with O_2 the reaction with the carrier and oxidase is so much more rapid that succinate is removed from the dehydrogenase surface rapidly enough to prevent oversaturation at this concentration.

For routine determinations, 10 mg. succinic acid in the 3 ml. (0·33 %) were used. Tubes were set up in duplicate or triplicate and two or more different amounts of tissue were used; usually amounts which would give reduction times 8–35 min. in the presence of succinate were chosen. In Table III examples of dehydrogenase determinations are shown and the results of determinations on a number of tissues are summarized in Table IV.

The value for Succ Q_{MB} is always much lower than Succ Q_{O_2} [see also Bach & Michlin, 1927; Wieland & Frage, 1929], which indicates that the reaction occurs less readily with methylene blue than with the natural carrier, cytochrome. The various tissues fall roughly into the same order according to dehydrogenase activities as they do according to their complete succinoxidase activities. However there is no constant relation between Succ Q_{MB} and Succ Q_{O_2}, a point which will be discussed further in a later section.

p-*Phenylenediamine oxidase*

Battelli & Stern [1912, 1] showed that all the tissues of higher animals have the power of oxidizing *p*-phenylenediamine and showed the variation of activity between different tissues. Several authors [Holmes, 1930; Penrose & Quastel, 1931; Quastel & Wheatley, 1932] have used the rate of oxidation of *p*-phenylenediamine as a measure of the "indophenol oxidase" or "respiratory ferment"

activities of various tissues. Quastel and his collaborators showed that the activity of the oxidase is not affected by destruction of the cell structure. In the next section it will be shown that since both the oxidase and cytochrome are concerned in the oxidation of *p*-phenylenediamine a true estimate of the full oxidase activity of tissues is not obtained unless excess cytochrome is present. However, the *p*-phenylenediamine oxidase, i.e. the oxidase plus cytochrome as present in the tissue suspensions, has been estimated in a number of tissues.

The procedure was similar to that for measuring succinoxidase activity. The *p*-phenylenediamine solution, freshly prepared each day, was added to both vessels of the manometer to compensate for the small autoxidation of the base (20–30 μl. per hr.). Tissue suspension was added only to the right-hand vessel. In a separate experiment the residual respiration of the tissue suspension at the concentration used was determined. This was always negligibly small and it was not deducted from the O_2 uptake observed with *p*-phenylenediamine since most of it probably takes place, like the *p*-phenylenediamine oxidation, through the mediation of the oxidase and cytochrome.

The rate of O_2 uptake increases rapidly with increasing *p*-phenylenediamine concentration up to about 0·6%, and the optimum concentration was found to be about 1%, further increase often producing a slight inhibition. In all estimations, therefore, the vessel fluid contained 30 mg. of the base in the 3 ml. In some cases the rate of *p*-phenylenediamine oxidation per unit weight of tissue increased somewhat with increasing concentration of tissue. Examples of this effect are shown in Table V. Duplicate determinations of O_2 uptake rates with *p*-phenylenediamine within a few hours often varied somewhat, but no constant effect of time of standing suspensions was noticed.

To express the rate of *p*-phenylenediamine oxidation, the term *p*-Phen Q_{O_2} will be used, where

$$p\text{-Phen } Q_{O_2} = \frac{\mu\text{l. } O_2 \text{ taken up in the oxidation of } p\text{-phenylenediamine}}{\text{hr.} \times \text{mg. tissue}}.$$

In Table VI the results obtained with various tissues are summarized. Again the order of the activity of the various tissues is about the same as for succinoxidase and the dehydrogenase. The activities found are several times higher than those given by Battelli & Stern [1912, 1] to whom only crude methods were available and who used tissues kept 5 hr. before mincing. As was noticed by Battelli & Stern, the rate of succinate oxidation by liver and kidney is somewhat higher than that of *p*-phenylenediamine (see Table VII). Both *p*-phenylenediamine and the succinate-dehydrogenase system react readily with cytochrome *c*, but it is possible that, with some tissues, structural arrangements on the tissue particles enable the latter system to react more rapidly than *p*-phenylenediamine with the insoluble cytochromes, *a* and *b*.

It is probable that both *p*-phenylenediamine and succinate-dehydrogenase can react with cytochromes *a* and *b* since in experiments in which the amount of soluble cytochrome was reduced by repeated washing of the tissue (brain, testis) the relative fall in O_2 uptake was small and about the same with either substrate.

The case of blood is exceptional. Whole rat blood diluted (and cytolysed) with water was used. With constant *p*-phenylenediamine concentration (30 mg. in 3 ml.), the O_2 uptake rate increased with increasing blood concentration up to about 200 mg. blood/3 ml.; further increase in blood concentration caused no further increase in O_2 uptake rate. The rate was steady, and it was quite low so that O_2 diffusion could not have been the limiting factor—increasing the surface by speeding up the shaking had no effect. It was noticed that the absorption

Table V. *Effects of tissue concentration and added cytochrome concentration on the* p*-phenylenediamine oxidation of rat tissues*

Tissue	Cytochrome added mg.	*p*-Phen Q_{O_2} (on moist wt.) mg. tissue in 3 ml. 3·1	5	6·3	10	12·5	20	25	37·5	40	50	75	100	150	200	300	400
Kidney	—	—	—	—	—	24·6	—	24·9	—	—	27·9	—	—	—	—	—	—
	1·6	—	—	—	—	68·5	—	68·8	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	21·8	—	—	23·7	—	—	—	—	—	—	—
	1·6	—	74·4	—	64·6	—	54·2	—	—	—	—	—	—	—	—	—	—
	3·2	—	72·0	—	66·7	—	57·8	—	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	12·5	—	—	12·8	11·9	—	—	—	—	—
	—	—	—	—	—	—	—	—	10·9	—	—	10·8	—	—	—	—	—
	1·6	—	—	—	50·5	—	46·1	—	38·0	—	—	—	—	—	—	—	—
	3·2	—	—	—	51·2	—	46·8	—	40·6	—	—	—	—	—	—	—	—
Heart	—	—	—	—	—	15·4	—	16·4	—	19·1	—	—	—	—	—	—	—
	—	—	—	—	—	23·2	—	25·6	—	—	—	—	—	—	—	—	—
	1·6	—	—	112·0	—	95·0	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	25·0	—	27·5	—	—	28·8	—	—	—	—	—	—
	1·5	97·8	—	94·9	—	91·8	—	—	—	—	—	—	—	—	—	—	—
	3·0	114·5	—	116·5	—	112·6	—	—	—	—	—	—	—	—	—	—	—
Brain	—	—	—	—	—	—	—	8·8	—	—	9·3	11·3	12·4	—	—	—	—
	0·7	—	—	—	—	—	—	15·0	—	—	14·6	—	—	—	—	—	—
	1·4	—	—	—	—	—	—	15·1	—	—	15·3	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	6·9	—	—	6·8	—	—	—	—	—
	1·5	—	—	—	29·5	—	29·7	—	24·4	—	—	—	—	—	—	—	—
	3·0	—	—	—	29·5	—	28·7	—	25·2	—	—	—	—	—	—	—	—
Testis	—	—	—	—	—	—	—	—	—	—	—	—	2·9	—	3·2	—	—
	0·7	—	—	—	—	—	—	—	—	—	—	—	6·9	—	6·5	—	—
	1·4	—	—	—	—	—	—	—	—	—	—	—	8·3	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	3·2	—	2·8	—
	1·5	—	—	—	—	—	—	—	12·7	—	—	11·1	—	9·2	—	—	—
	3·0	—	—	—	—	—	—	—	13·1	—	—	11·5	—	10·0	—	—	—
Lung	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·1	—	1·0
	1·0	—	—	—	—	—	—	—	—	—	—	—	—	—	5·7	—	—
	2·0	—	—	—	—	—	—	—	—	—	—	—	—	—	6·8	—	—
Skeletal muscle	—	—	—	—	—	—	—	—	—	—	—	2·8	—	—	3·5	—	—
	0·5	—	—	—	—	—	—	—	—	—	—	9·3	—	—	9·3	—	—
	1·0	—	—	—	—	—	—	—	—	—	—	10·1	—	—	10·1	—	—
Spleen	—	—	—	—	—	—	—	—	—	—	—	—	1·6	—	1·4	—	—
	0·7	—	—	—	—	—	—	—	—	—	—	—	5·6	—	—	—	—
	1·4	—	—	—	—	—	—	—	—	—	—	—	6·7	—	—	—	—
Jensen sarcoma	—	—	—	—	—	—	—	—	—	—	—	—	—	3·3	—	3·9	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	1·8	—	1·6	—
	1·5	—	—	—	—	—	—	8·1	—	—	6·6	—	5·2	—	—	—	—
	3·0	—	—	—	—	—	—	8·0	—	—	7·4	—	5·8	—	—	—	—
Phila. 1 sarcoma	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·8	—	0·8
	1·0	—	—	—	—	—	—	—	—	—	—	—	—	—	4·6	—	—
Blood	—	—	—	—	—	—	—	6·6	—	—	4·6	2·8	—	1·5	—	0·7
	1·0	—	—	—	—	—	—	6·9	—	—	—	—	—	1·5	—	—

Table VI. p*-Phenylenediamine oxidase activities of rat tissue suspensions*

Tissue	*p*-Phen Q_{O_2} On moist wt.	Mean on dry wt.
Kidney	16·4, 17·7, 21·9, 20·0, 24·7, 23·7	91
Liver	11·9, 10·9, 10·4, 13·6, 14·1, 10·2, 11·9, 10·9	40
Heart	20·5, 19·1, 23·4, 16·4, 18·8, 23·5, 27·5	96
Brain	8·4, 8·1, 8·9, 10·7, 8·4, 9·8, 9·8, 9·3, 6·8	43
Testis	3·0, 2·8, 3·1, 3·5, 3·2, 3·4, 2·5, 2·5, 3·1	24
Lung	1·1, 1·2	5·8
Retina (Ox)	1·5	13

Tissue	*p*-Phen Q_{O_2} On moist wt.	Mean on dry wt.
Skeletal muscle	2·9, 1·8	10
Spleen	1·5, 1·3	6·2
Pancreas	0·5, 0·3	1·0
Jensen sarcoma	2·2, 2·9, 3·3, 1·3, 1·7	14
Flexner-Jobling	1·1, 0·6	5·2
Walker No. 256	0·5, 0·3, 0·5, 0·4,	2·6
Phila. No. 1	0·8, 0·7	4·2
Blood (maximum observed)	6·6	124

bands of oxyhaemoglobin could be observed with the higher amounts of blood, they were faint at the limiting concentration, and completely absent in the range where O_2 uptake was proportional to blood concentration. In the latter solutions it appeared that the haemoglobin was completely destroyed; no methaemoglobin band in the red could be seen nor was reduced haemoglobin regenerated on the addition of hydrosulphite. Addition of cytochrome had no effect on the oxidation. When the concentration of blood was kept constant at a high value (450 mg./3 ml.), the O_2 uptake increased greatly with increasing *p*-phenylenediamine concentration tending to a maximum rate at a very high concentration of the base. It seems probable that the actual catalyst of the oxidation is a breakdown product of haemoglobin formed by a reaction between haemoglobin and *p*-phenylenediamine, and the amount formed is a function of both haemoglobin and *p*-phenylenediamine concentrations. Entirely similar results were obtained with crystalline horse haemoglobin; these results are shown in Figs. 1 and 2. The haemoglobin solutions were estimated by the ferricyanide method.

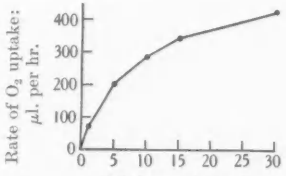

Fig. 1.

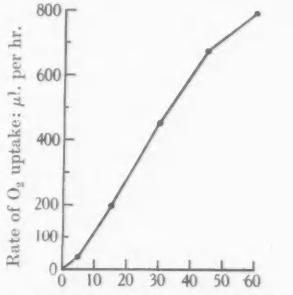

Fig. 2.

Fig. 1. Effect of varying HbO_2 concentration, with constant (30 mg.) *p*-phenylenediamine.

Fig. 2. Effect of varying *p*-phenylenediamine concentration, with constant (30 mg.) HbO_2.

Cytochrome

No attempt has been made to estimate cytochrome in the tissues directly, but some information about the quantitative relations of oxidase, cytochrome and dehydrogenase in tissue suspensions has been obtained by experiments in which known amounts of cytochrome were added.

Solutions of pure oxidized cytochrome *c* were obtained from ox heart muscle by the simple method of Keilin & Hartree [1937]. The cytochrome-iron content was estimated[1] by the $\alpha\alpha$-dipyridyl method [Hill & Keilin, 1933].

The experiments were carried out exactly as in the previous sections with 0·1–0·4 ml. of the cytochrome solution added to the medium in the vessels. O_2 uptake rates of various tissues with *p*-phenylenediamine and with succinate in the presence and absence of added cytochrome *c* are shown in Table VII.

Effect on p-*phenylenediamine oxidation.* Formerly it was believed that the oxidation of *p*-phenylenediamine, or the "Nadi" reagent, was catalysed directly by the oxidase, but it will be seen that the oxidation of *p*-phenylenediamine is greatly accelerated by the addition of cytochrome with all the homogenized

[1] These estimations were kindly made for us by Dr H. K. Alber.

Table VII. *Results of determinations, on the same sample of rat tissue, of the rates of oxidation of succinate and* p-*phenylenediamine, separately and together, with and without added cytochrome*

Homogenized suspensions used, unless otherwise stated. Activities calculated on moist weights.

Tissue	Cytochrome mg. in 3 ml.	Succ Q_{MB}	Succ Q_{MB} / Succ Q_{O_2}	Succ Q_{O_2}	*p*-Phen Q_{O_2}	*p*-Phen Succ Q_{O_2}
Kidney	—	—	—	22·6	16·4	24·0
	—	2·2	0·09	18·9	17·7	26·1
	2·0	—	—	17·4	36·4	34·9
Liver	—	—	—	13·3	10·9	13·1
	—	1·8	0·13	13·4	13·2	13·2
	0·7	—	—	13·2	19·1	12·6
	1·4	—	—	—	21·3	—
	—	1·4 ±	0·10	13·8	11·0	—
	0·5	—	—	14·4	23·0	14·1
Heart	—	—	—	13·0	23·4	26·0
	0·7	—	—	24·0	35·5	—
	1·4	—	—	23·8	42·2	43·3
	—	3·4	0·28	12·1	16·4	18·1
	1·4	—	—	17·8	43·4	28·6
Brain	—	0·81	0·20	4·1	8·4	14·3
	0·7	—	—	4·2	14·9	13·9
	—	—	—	4·0	8·9	14·4
	1·4	—	—	4·6	15·7	15·4
	3·5	—	—	—	15·8	—
Testis (Teased)	—	—	—	0·94	4·3	3·1
Testis (Homogenized)	—	—	—	1·2	2·8	3·8
Testis (Teased)	—	—	—	0·69	5·0	3·5
	0·7	—	—	0·73	5·2	4·1
Testis (Homogenized)	—	0·16	0·09	1·7	3·1	4·8
	0·7	—	—	1·7	6·7	4·8
	1·4	—	—	—	8·3	—
Lung	—	0·37	0·42	0·86	1·1	1·2
	2·0	—	—	1·7	6·8	6·0
Skeletal muscle	—	0·6	0·32	1·9	2·9	2·6
	1·0	—	—	2·8	10·1	5·7
Spleen	—	0·31	3·4	0·09	1·5	1·63
	0·7	—	—	0·85	5·6	2·3
	1·4	—	—	1·3	6·8	—
	—	—	—	0·04	—	—
	0·7	—	—	1·0	—	—
	1·4	—	—	1·7	—	—
Jensen sarcoma	—	0·22	—	2·0	2·9	3·6
	—	0·20	0·10	2·0	3·3	4·3
	1·0	—	—	1·9	4·7	4·8
Flexner-Jobling	—	—	—	2·0	1·1	1·3
	—	—	—	0·4	0·6	—
	1·6	—	—	1·8	5·0	—
Walker No. 256 carcinoma	—	—	—	0·16	0·51	0·4
	1·6	—	—	1·2	3·8	—
	—	0·06	0·7	0·09	0·27	0·37
	0·7	0·06	—	—	0·70	0·52
	1·4	—	—	0·20	0·96	—
Philadelphia No. 1 sarcoma	—	0·19	0·48	0·40	0·76	0·79
	1·0	—	—	1·09	4·6	3·3
	2·0	—	—	0·91	5·8	—
	—	—	—	0·0	0·65	—
	1·6	—	—	0·8	4·0	—
Pancreas*	—	—	—	0·04	0·49	—
	1·6	—	—	0·05	0·48	—

* Results with pancreas are doubtful. It is possible that enzymes are destroyed in suspensions of this tissue. Also the suspensions become acid fairly rapidly.

tissues so far tried; Ogston & Green [1935] found the same with an enzyme preparation from heart. The reduction of oxidized cytochrome *c* by a solution of *p*-phenylenediamine occurs immediately and can be readily observed with a hand spectroscope. The Nadi reagent also reduces cytochrome, and with a tissue suspension in an open test tube with added Nadi reagent the development of the blue colour throughout the solution occurs much more rapidly if a little oxidized cytochrome is added. It seems definite, therefore, that these oxidations take place through the mediation of cytochrome, and the oxidation which occurs without added carrier may be due to the cytochrome already present in the tissue suspension. (Keilin & Hartree [1938] have recently demonstrated the same points very thoroughly.)

From Table V it will be noticed that with added cytochrome the rate of *p*-phenylenediamine oxidation per unit weight of tissue increases toward a maximum with decreasing concentration of tissue and increasing concentration of cytochrome: i.e. the maximum oxidase activity is shown when the ratio of cytochrome concentration to tissue concentration is high. Keilin [1930] showed similar effects of cytochrome and enzyme concentration in the oxidation of cysteine by preparations of heart muscle indophenol oxidase.

It follows that to obtain a maximum estimate of the oxidase content of a tissue it is necessary to add excess cytochrome with the *p*-phenylenediamine, using low concentrations of tissue. In this way the oxidase activity of rat heart, for instance, is found to be equivalent to the remarkably high figure of $Q_{O_2}=500$ approximately (calculated on the dry weight of tissue). Even these high figures possibly do not represent the absolute maximum for the tissues, since the process of homogenizing probably does not result in the rupture of all cells and the extra cytochrome would not penetrate into intact cells. But it is seen that with every tissue the oxidase activity is considerably higher than is necessary to account for the whole normal respiration as measured by the tissue slice technique. In Table VIII estimates of approximately the full oxidase activities of a few tissues are given.

Table VIII. *Estimates of the full oxidase activities of rat tissues. Rates of O_2 uptake of tissue suspensions in the presence of* p-*phenylenediamine and excess of cytochrome* c

	p-Phen Q_{O_2}			*p*-Phen Q_{O_2}	
Tissue	On moist wt.	Mean on dry wt.	Tissue	On moist wt.	Mean on dry wt.
Kidney	68·8, 72·0	288	Spleen	6·8, 7·8	32
Liver	51·2	167	Muscle	10·1, 8·1	38
Heart	114·5, 112·0	506	Jensen sarcoma	8·0, 5·7	43
Brain	29·5	134	Flexner-Jobling carcinoma	5·0	28
Testis	13·1	106	Walker No. 256 carcinoma	1·0, 3·8, 1·3	15
Lung	6·8, 7·8	31	Phila. No. 1 sarcoma	4·0, 5·8	29
			Ox retina	10·6	88

With tissue in which the cells have not been extensively broken up by homogenization the results are somewhat different. This is seen (Table VII) in experiments with rat testis which was merely pulled apart into loose bunches of tubules. With this "teased" testis the *p*-phen Q_{O_2} was higher than with the homogenized tissue but addition of cytochrome scarcely affected the rate. Apparently the oxidation of the *p*-phenylenediamine takes place inside the cell

where there is a certain concentration of cytochrome; added cytochrome, not being permeable into the cells does not affect the rate. The homogenized tissue has a lower original *p*-phen Q_{O_2} due, perhaps, to the dilution of the cytochrome from the broken cells, but added cytochrome can now come into contact with the oxidase and so can raise the O_2 uptake rate even above that found in the intact cells with their limited content of carrier.

Effect of succinate oxidation. Results of experiments on the O_2 uptake of tissue suspensions in the presence of succinate with and without the addition of cytochrome are shown in Table VII.

With spleen only a trace of succinoxidase activity was found directly, although the experiments on methylene blue reduction showed the presence of dehydrogenase. It was therefore supposed that the lack of O_2 uptake with succinate was due to lack of carrier, and, as expected, it was found that on the addition of cytochrome the O_2 uptake was increased 10–40 times. With heart there is also a considerable acceleration (50–80%) of succinate oxidation on adding cytochrome, which shows that, while this tissue contains cytochrome, the amount in a suspension is not enough to carry the rapid H transfer which the high dehydrogenase activity (Succ $Q_{MB} = 3{\cdot}1, 4{\cdot}1$) of the tissue is capable of activating.

With brain, testis, kidney and liver the addition of cytochrome does not appreciably affect the succinate oxidation, there being evidently enough cytochrome present to effect H transfer as fast as the dehydrogenase present can "activate" it. It will be noticed that the rate of methylene blue reduction by suspensions of these three tissues is particularly low compared with the rate of O_2 uptake, i.e. the ratio Succ Q_{MB}/Succ Q_{O_2} is particularly low. In any tissue in which this ratio exceeds about 0·2, it is found that the dehydrogenase activity exceeds the capacity of the cytochrome present to transfer all the H activated; the limiting factor is the concentration of cytochrome (oxidase always being present in excess) and accelerated O_2 uptake is obtained by adding cytochrome. It should be mentioned that experiments showed that the addition of cytochrome had no effect on the time of reduction of methylene blue; no carrier is required when methylene blue replaces O_2 and oxidase.

The rate of O_2 uptake with p*-phenylenediamine plus succinate*

With a number of tissues the O_2 uptake rate was determined in the presence of optimal concentrations of both succinate and *p*-phenylenediamine. The term ${}^{p\text{-Phen}}_{\text{Succ}}Q_{O_2}$ is used to express these rates. Results are given in Table VII. It will be seen that with the majority of homogenized tissues, in the absence of added cytochrome, *p*-phenylenediamine and succinate together gave an O_2 uptake greater than that obtained with either substrate separately. In fact with several tissues, notably brain and testis, the effect was equal to or greater than the sum of the effects of either substrate separately. With other tissues, notably liver, the "additive" effect is not apparent. Battelli & Stern [1912, 1, 2] noticed this "additive" effect with brain and its absence in liver, and considered it probable that the mechanisms for the oxidation of *p*-phenylenediamine and of succinate were not identical in brain. However, as can be seen in Table VII, the "additive" effect is not confined to brain; it occurs in most tissues, varying from an effect in excess of simple addition of the O_2 uptakes of the two substrates separately, in some experiments with brain, to complete absence of summation with liver. In view of the well-known effects of inhibitors such as HCN, it seems most probable that the oxidations of both *p*-phenylenediamine and succinate take place almost exclusively through cytochrome and indophenol oxidase. Many

experiments were tried to explain the "additive" effect until it was found that the oxidations of *p*-phenylenediamine by brain and testis could be accelerated not only by adding succinate but also by fumarate, malonate or acetate, none of which substances was itself rapidly oxidized by the suspensions in the absence of *p*-phenylenediamine. With liver, fumarate and malonate had little effect on the oxidation of *p*-phenylenediamine. (Acetate increased the O_2 uptake of liver with *p*-phenylenediamine markedly. This result was repeatedly observed but cannot yet be explained.) Some results are shown in Table IX.

Table IX. *Effects of Na salts of acids on* p-*phenylenediamine oxidation*

Salt added equivalent to 0·15 *M* Na in each case.

	Cyto-chrome	*p*-Phen Q_{O_2} on moist wt.				
		No addition	Succinate	Fumarate	Malonate	Acetate
Brain	–	9·8	16·6	14·7	15·3	14·4
	+	23·2	18·0	15·6	16·1	17·4
Testis	–	2·5	3·6	3·2	3·5	3·2
	+	7·4	4·0	3·3	3·4	3·8
Liver	–	11·9	15·0 (16·8)*	12·4	13·9	18·5
	+	40·0	23·9	18·3	19·3	30·9

* Succinate without *p*-phenylenediamine, i.e. Succ Q_{O_2}.

It seems most likely that the "additive" effect is in reality simply a salt effect of the Na salts of the acids added.

That succinate oxidation is not concerned in the observed stimulation of O_2 uptake of brain and testis with *p*-phenylenediamine, is shown by the following experiments. (1) Estimations of succinate indicated that, in the presence of *p*-phenylenediamine, succinate oxidation was inhibited. (Succinate was determined by the manometric succinoxidase method after removing the diamine with permanganate and extraction of the succinate with ether.) (2) Labes & Krebs [1935] and Potter & Elvehjem [1937] showed that selenite in low concentration inhibits succinic dehydrogenase and we have confirmed this both on O_2 uptake and methylene blue reduction. Selenite causes a small, 4–20%, acceleration of *p*-phenylenediamine oxidation, and the high O_2 uptake of *p*-phenylenediamine plus succinate is also slightly further *increased* by selenite. If oxidation of succinate caused the additional O_2 uptake with *p*-phenylenediamine plus succinate, selenite would *reduce* the total O_2 uptake. Similar results were obtained with malonate which also is known to inhibit succinic dehydrogenase [Quastel & Wooldridge, 1928; Quastel & Wheatley, 1931]. (3) Finally, the formation of dark oxidation products of *p*-phenylenediamine with brain suspensions is appreciably more rapid when salts of succinate and other acids are added, as can be readily observed in test tube experiments. With liver these salts seem to delay the darkening. The inhibitory effect of the salts on the O_2 uptake with *p*-phenylenediamine plus cytochrome, mentioned below, can also be observed.

Battelli & Stern [1912, 2] noted that NaCl solutions up to 0·1–0·17 *M* increased the rate of *p*-phenylenediamine oxidation by brain mince. The succinate (presumably $C_4H_4O_4Na_2$, $6H_2O$) in the concentration used by them [1912, 1] corresponded to 0·12 *M* Na and so should be expected to accelerate *p*-phenylenediamine oxidation purely as a salt effect; and in fact it did so to just about the same relative extent (31%, 35%) as they found with NaCl in their next paper [1912, 2]. With liver mince, weak NaCl solutions, 0·05 *M* increased the rate of oxidation of *p*-phenylenediamine but with stronger solutions, >0·1 *M*, the rate decreased again. With the amount of sodium succinate used by them it was to be expected that only a small increase in rate of O_2 uptake would be observed.

The mechanism of this salt effect is not clear. It will be noticed (Tables VII and IX) that with all the tissues the O_2 uptake rate with *p*-phenylenediamine plus excess cytochrome is usually lowered on the addition of succinate or other

salts. The salts may improve the adsorption of cytochrome on the oxidase, in brain and testis particularly, and so increase its rate of oxidation, thus increasing the O_2 uptake rate when cytochrome is the limiting factor; but when excess of cytochrome is present the activity of the oxidase is the limiting factor and salts may have a depressing effect on this. The difference between brain and liver, at the two extremes, may be due to differences in the colloidal behaviour of the tissue particles with which the oxidase is associated. Further work on these points is in progress.

Note. Breusch [1937], in a paper which appeared after the completion of this work, reports a similar distribution of succinoxidase activity, the activity being measured by the formation of fumarate $\rightleftharpoons$ malate. He did not observe the excess of cytochrome oxidase in the tissues.

Summary

1. Using tissues reduced to fine suspensions by Potter & Elvehjem's [1936] method, conditions have been worked out under which the approximate maximum rates of oxidation of succinate and *p*-phenylenediamine can be observed. A method of applying the Thunberg methylene blue reduction technique for the determination of succinic dehydrogenase in tissue suspensions is described. Using these methods estimates have been obtained of the activity of the complete succinoxidase system, the indophenol oxidase and the succinic dehydrogenase in various tissues.

2. The complete system, "succinoxidase", is extremely active in kidney, liver and heart. Brain, testis, skeletal muscle, lung, adrenals and retina have moderate to low activities. Thymus, pancreas, spleen and blood, have practically no activity. Cancer tissues vary; Philadelphia No. 1 sarcoma, Walker 256 carcinoma (usually) and certain spontaneous mammary carcinomata (rat) are inactive, while Jensen sarcoma, Flexner-Jobling and one example of Walker 256 carcinoma showed definite, though low, activities.

3. The oxidation of succinate by methylene blue is much slower than by O_2, and there is no constant relation in different tissues between the rate of reduction of methylene blue and the rate of O_2 uptake. But in general the tissues fall into the same order when arranged according to their activity measured in either way.

4. The tissues fall into roughly the same order according to their activity in oxidizing *p*-phenylenediamine.

5. The oxidation of *p*-phenylenediamine is effected by the indophenol oxidase through cytochrome and it is only in the presence of excess cytochrome that an estimate of the full oxidase activity of the tissues can be obtained. On adding excess of cytochrome *c*, the oxidation of *p*-phenylenediamine is considerably increased in all homogenized tissues. In this way it is found that the activity of the tissues is in all cases considerably higher than is necessary to account for the whole respiration of the normal tissue.

Since cytochrome does not penetrate cells, the effect of adding cytochrome is not apparent in incompletely disintegrated tissue.

6. Cytochrome is a necessary link in the complete succinoxidase system. In some tissues, the ratio between rate of methylene blue reduction (i.e. the dehydrogenase estimate) and rate of O_2 uptake is comparatively high. In these cases the O_2 uptake rate with succinate can be increased to a maximum value by adding cytochrome *c*.

7. In the presence of optimal concentrations of both *p*-phenylenediamine and succinate, many tissues, notably brain and testis, give O_2 uptake rates

greater than with either substrate separately. This effect seems to be due, not to a difference in oxidases concerned with the two substrates, but to a salt effect of the Na succinate on the oxidase-cytochrome activity; similar results were obtained with fumarate, malonate, and acetate. With liver the "additive" effect is absent. With all the tissues the high rate of *p*-phenylenediamine oxidation in the presence of added cytochrome is lowered on the addition of succinate and other salts.

8. Blood catalyses the oxidation of *p*-phenylenediamine strongly but the mechanism is quite different from that in other tissues. The catalyst concerned seems to be a breakdown product of haemoglobin produced by reaction with the diamine. Cytochrome addition has no effect.

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CLXXXVIII. THE RELATION BETWEEN VITAMIN C AND ADRENALINE

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It was shown by Daoud & Ayyadi [1936] that the rate of glycogenolysis caused by adrenaline, under constant conditions, ran parallel with the degree of saturation of the body with vitamin C. Two alternative suggestions were offered to explain this parallelism. The first was that the degree of saturation of the body with vitamin C determines directly the rate of glycogenolysis, though hyperglycaemia and glycosuria are produced in states of saturation with the vitamin only when the peripheral utilization of the blood sugar is interfered with by adrenaline. The second suggestion was that the vitamin in the reduced form protects adrenaline from rapid oxidation and thus increases glycogenolysis.

As regards the second suggestion, such a relationship has been suggested by different investigators owing to the co-existence of both substances in the suprarenal and to the reduction in the amount of both stated by some to occur in scurvy. The stabilizing effect of the vitamin on adrenaline may be inferred from the work of Szent-Györgyi [1928], Heard & Raper [1933], and Euler & Klussmann [1933]. More recently Heard & Welch [1935], working on the Ringer-Locke perfusate of the suprarenal glands, found that as long as a small amount of reduced ascorbic acid remained in the perfusate there was no diminution in its pressor activity. They also found that the O_2 uptake of the perfusate was characteristic of an oxidation-reduction system. They stated that the perfusate contained neither sulphydryl compounds nor any reducing substances known to occur in the gland other than ascorbic acid, but did contain an agent which minimized the rate of autoxidation of ascorbic acid.

Against the results of these investigators may be set those of Deutsch & Schlapp [1935] who found a reduction in the adrenaline content of the suprarenals of guinea-pigs in inanition but no significant change in their ascorbic acid content, provided that sufficient green food were given in the diet. It thus appeared that inanition was an important factor in the reduction of the adrenaline content of the suprarenals in scorbutic animals. They came to the conclusion that there was no close physiological relation between ascorbic acid and adrenaline.

The relation of these two substances, even in the suprarenal which is rich in both, thus remains obscure. The suprarenal is concerned in the elaboration and storage of adrenaline and it is natural to suppose that it is provided with some system which protects this hormone from destruction; alternatively, it may be deficient in the agents elsewhere responsible for the destruction of adrenaline. This view is strengthened by the fact that the liver is also an organ with a high vitamin C content, yet, according to Markowitz & Mann [1929], Elliott [1905] and Giragossintz & Mackler [1929], the liver is particularly active in the destruction of adrenaline. The work on the suprarenal or its perfusate will

therefore give no clue to the part which the vitamin alone plays in its physiology, especially if we bear in mind, from the data reported by Wiltshire [1931], Kellie & Zilva [1935], Mawson [1935], Hopkins & Morgan [1936] and Heard & Welch [1935], that most of the substances and tissue extracts which exert a protective effect on the vitamin are also protective for adrenaline, and that substances which protect the vitamin are found in the perfusate of the suprarenals. These protective substances are widely distributed in the body, so, even if we admit a protective influence of the vitamin on the adrenaline in the suprarenals or in pure mixtures of the two substances, it will still be questionable whether this protective effect is of particular significance in the body as a whole, where numerous other protective substances are present and where certain organs appear to be more active in the destruction of adrenaline than others.

These considerations made it desirable to find out how far the work done on isolated suprarenals or on adrenaline *in vitro* applied to the functions of vitamin C in the body as a whole and to decide whether the effect of the vitamin in augmenting the rate of glycogenolysis caused by adrenaline is due to a stabilizing effect of the vitamin on adrenaline or is due to a direct glycogenolytic effect of the former.

During the course of this investigation, the effect of the vitamin on the blood pressure was studied in pithed animals.

Experimental

Since experiments carried out *in vitro* or on isolated organs are suspected of being unhelpful as a criterion for the state of events in the body as a whole, it was decided to work on the whole animal. We first compared the effects produced by certain doses of adrenaline injected before and after the gradual increase of the vitamin C content of the body.

A pithed cat was injected intravenously with different doses of 1/10,000 adrenaline hydrochloride solution and the maximum rise in blood pressure and the duration of the rise caused by each dose were recorded. Ascorbic acid (B.D.H.) was then given and the effects of the previous doses of the adrenaline hydrochloride were again tried. Ascorbic acid and adrenaline hydrochloride were then administered alternately to determine the effect of adrenaline after different amounts of ascorbic acid had been administered.

It was noticed that ascorbic acid injected in doses of 100 mg. into a 2700 g. cat had no effect on the blood pressure, but when the dose was increased to 200 mg. a rapid drop in blood pressure occurred. The maximum drop was 24–28 mm. and lasted for 93–95 sec. Reinjection of the same doses of adrenaline which had been given before the administration of ascorbic acid caused a much smaller and less sustained rise in blood pressure than before. Adrenaline injected in considerably larger doses than those given before the ascorbic acid administration caused a rise of much smaller extent and shorter duration than would be expected. Simultaneous administration of the two substances produced the same small effects.

The effect of ascorbic acid in lowering the blood pressure might have been due to its acidity rather than to any specific influence of the vitamin. The observation that adrenaline had less effect on the blood pressure after the ascorbic acid injections might possibly be explained by the fact that the acidity caused the production of substances which antagonized the action of adrenaline. The experiment was therefore repeated on a pithed cat weighing 2·5 kg., the ascorbic acid being neutralized with $N/100$ NaOH immediately before injection. In this experiment the magnitude and duration of the rise in blood pressure

caused by adrenaline were the same after as before the administration of the neutralized ascorbic acid; further, doses of 200 mg. of the neutralized acid caused neither rise nor fall in blood pressure as shown in Table I.

Table I

Substances in the order of injection	Blood pressure (mm. Hg)		Duration of rise or fall sec.
	Maximum rise	Maximum fall	
0·1 ml. adrenaline*	52	—	130
0·2 ,,	96	—	165
0·1 ,,	58	—	130
0·2 ,,	91	—	190
0·1 ,,	54	—	145
0·2 ,,	88	—	165
100 mg. ascorbic acid	—	—	—
100 ,, ,,	—	—	—
200 ,, ,,	—	—	—
0·2 ml. adrenaline	91	—	165
0·1 ,,	56	—	132
100 mg. ascorbic acid	—	—	—
100 ,, ,,	—	—	—
100 ,, ,,	—	—	—
100 ,, ,,	—	—	—
100 ,, ,,	—	—	—
0·1 ml. adrenaline	52	—	135
0·1 ,,	58	—	140
0·2 ,,	89	—	187
0·1 ml. adrenaline + 100 mg. ascorbic acid	57	—	137
200 mg. ascorbic acid	—	—	—
200 ,, ,,	—	—	—
0·2 ml. adrenaline	88	—	190
0·1 ,,	56	—	140
0·1 ,,	53	—	136
0·1 ,,	57	—	145
0·2 ,,	90	—	185

* 1/10,000 adrenaline hydrochloride solution.

The injection of a total quantity amounting to 1·4 g. of ascorbic acid, adjusted to the physiological *p*H, into a cat of 2·5 kg. body weight neither augmented nor prolonged the effect of adrenaline on the blood pressure.

The above results, although instructive, do not disprove the existence of a protective effect of ascorbic acid on adrenaline, since the destruction of adrenaline in the body appears, from the work of Weiss & Harris [1904], not to coincide with the return of the blood pressure after the injection of adrenaline to its original level. These investigators found that when a cat was injected with adrenaline the transfusion of its blood taken after the blood pressure had fallen to its original level into a second cat caused a rise of blood pressure in the latter. We therefore attempted to find out the time necessary for the destruction of a certain amount of adrenaline in animals saturated with the vitamin and in normal controls in the following manner.

Two cats were chosen of exactly the same weight, 2800 g., and were kept on the same diet. One of the cats received 7 daily injections of 70 mg. ascorbic acid neutralized with Na_2CO_3 immediately before injection. On the 8th day the two cats were anaesthetized with intraperitoneal injections of dial. 5 ml. samples of arterial blood were taken from each cat and injected alternately into a third pithed cat and the small rise in blood pressure was noted in each case.

Each of the two cats was injected 3 times intravenously with 1 ml. adrenaline hydrochloride 1/1000, allowing 5 min. between each injection. Samples of 5 ml. of arterial blood taken after definite intervals of time had elapsed since the last adrenaline injection in each case were immediately injected into the femoral vein of the pithed cat. The rise in blood pressure above the initial rise caused by the blood of each cat before the adrenaline injection was noted in each case. The transfusion of blood was continued till the samples of blood taken from each cat caused no greater rise than that caused by the blood of this particular cat before it had received adrenaline. The time between the last injection of adrenaline and the disappearance of its effect on the blood pressure of the pithed cat was taken as the time necessary for the destruction of adrenaline in the cats under experiment as indicated in Table II.

Table II

Time after last adrenaline injection min.	Rise in blood pressure above initial mm. Hg	
	Control cat	Cat injected with ascorbic acid
5	108	—
20	86	47
40	52	32
60	30	20
80	7	5
90	4	Nil
95	Nil	—

It will be noticed that in this experiment the rise in the blood pressure of the pithed cat caused by the blood of the cat injected with ascorbic acid was less than in the case of the control cat, but this was not always so. The main observation is that the time taken by the two cats to destroy the same amount of adrenaline is practically the same, being, in fact, 5 min. less in the case of the cat previously injected with ascorbic acid. In another experiment a total dose of 2 ml. of 1/1000 adrenaline hydrochloride was injected into each of two cats weighing 2700 g.; the destruction of adrenaline took place in 70 min. in both cases and there was no appreciable difference in the magnitude of rise of blood pressure caused by the two bloods. These two experiments are especially recorded because the blood taken from the two cats before adrenaline injections did not show a depressor effect on the pithed cat, as sometimes happened, and the experiments were successfully carried out to the end.

The above result seems to indicate that, in the intact animal, ascorbic acid has no protective effect on adrenaline. Since it has been shown by Sandiford [1920] and by Cori & Cori [1928] that adrenaline causes a rise in metabolism, it was decided to compare the time necessary for the disappearance of the effect of adrenaline on the metabolism of rats saturated with ascorbic acid with the time necessary in the case of unsaturated rats. For this purpose a number of male rats were chosen weighing 250–255 g. They were divided into two batches and all were kept on a scorbutic diet for 7–10 days. Each of the members of one batch then received daily injections of 7 mg. of ascorbic acid for 3–9 days before the adrenaline administration. Food was removed at 4 p.m. on the day preceding an experiment. On the days of experiments, one pair of rats taken from each batch was injected with 0·1 ml. physiological saline and the O_2 consumption by the pair was then determined every 20 min. in a closed system.

In the first 20 min. a high consumption was always noticed, which was undoubtedly due to the effect of handling; the observations were continued until steady consumptions were attained in the subsequent 20 min. periods. Usually the amount of O_2 consumed in the second or third 20 min. agreed with the subsequent readings. The constant amount of O_2 consumed was taken as the basal amount for the pair in 20 min. Each rat then received an injection of 0·1 ml. 1/1000 adrenaline hydrochloride solution and the readings were again taken until the quantity of O_2 consumed in 20 min. returned to the basal amount. The time between the adrenaline injection and the end of the last 20 min. during which there was still a rise in O_2 consumption above the basal, was taken as an estimate of the time necessary for the wearing away of the effect of a definite amount of adrenaline in the bodies of the saturated and unsaturated rats. In a very few cases, continuous fluctuations in the O_2 consumption in the 20 min. periods were noted, especially after adrenaline injections, but such results were rejected. Many experiments were performed in the manner described; for the sake of brevity, the results obtained with six pairs of rats are given in Table III.

Table III

Diet	Time for disappearance of adrenaline effect (min.)					
	I	II	III	IV	V	VI
Scorbutic	140	120	160	140	120	120
Scorbutic + ascorbic acid injections	120	120	140	140	160	140

The time necessary for the disappearance of the effect of the adrenaline in the two groups was of the same order and varied from 120 to 160 min. This result agreed with that of the blood transfusion experiments in suggesting that ascorbic acid had no protective effect on adrenaline *in vivo*.

It has already been mentioned that Daoud & Ayyadi [1936] gave two alternative suggestions in explanation of the parallelism between the degree of saturation of the body with vitamin C and the rate of glycogenolysis caused by adrenaline. The present work rules out the suggestion of a protective effect of vitamin C on adrenaline.

Effect of vitamin C on adrenaline in vitro

Adrenaline is known to undergo rapid autoxidation in atmospheric oxygen. Although there is a tendency at present to consider that adrenaline is destroyed in the body by enzymes, yet the rapidity of autoxidation suggests at least that this may be partially responsible for the destruction. This, together with the apparent similarity between the mechanisms of autoxidation and some of the enzymic oxidations, made it desirable to examine the effect of the vitamin, when isolated from other systems, on the oxidation of adrenaline.

Heard & Welch [1935], in their work on the effect of atmospheric oxygen on the perfusate of the suprarenals already referred to, concluded that the stabilization of adrenaline was effected by means of an oxidation-reduction system, and that ascorbic acid in the reduced form was the agent responsible for the protection of the catechol group of adrenaline from oxidation to *o*-quinone. When the ascorbic acid was completely oxidized, irreversible oxidation of adrenaline, manifested by the appearance of a red coloration and diminution in pressor activity, commenced; thereafter the rate of adrenaline oxidation was dependent on the amino-acid content of the perfusate.

It was planned to study the problem in solutions containing mixtures of the two pure substances. Since adrenaline is more stable in acid than in neutral or alkaline solutions, the effect of the acidity of ascorbic acid was eliminated by buffering the solutions at *p*H 7 with phosphate. The relative proportions of adrenaline hydrochloride and ascorbic acid in the solutions were of the order reported by Heard & Welch to be present in some of the perfusates of the suprarenals; the ratio of ascorbic acid to adrenaline was increased in some of the experiments for the purpose of comparison.

It is generally accepted that the appearance of the red colour in adrenaline solutions is an indication of irreversible oxidation of adrenaline and is accompanied by diminution in pressor activity. The red-coloured substance was considered by Green & Richter [1937] to be adrenochrome, which is inactive as a vasoconstrictor.

In the present work the commencement of irreversible oxidation was judged at first by the appearance of this red colour. Equal volumes of a solution of adrenaline hydrochloride in phosphate buffer were placed in a number of bottles of 250 ml. capacity. The bottles were all brown in colour with the exception of one colourless bottle which was used for comparing the effect of light on the decomposition of adrenaline. The total volume of solution in each bottle was always 100 ml.; all solutions were buffered at *p*H 7 and contained the concentrations of adrenaline alone or adrenaline and ascorbic acid indicated in Table IV. In addition, control experiments were made in which only buffered ascorbic acid (B.D.H.) was included. All bottles were tightly stoppered with glass stoppers and were shaken every now and then for the same length of time on a mechanical shaker to facilitate oxidation by the air enclosed above the liquid. After each period of shaking the brown bottles were placed in the dark and the colourless bottle was placed in the light.

In most of the experiments the reddish tinge appeared in the different solutions in the following order: adrenaline alone kept in light, simultaneously in the different solutions of adrenaline + ascorbic acid, adrenaline alone kept in dark; no red colour appeared in the solutions containing ascorbic acid alone. In some cases, however, the appearance of the red tinge in the solutions containing adrenaline and ascorbic acid together took place at the same time as in the solutions of adrenaline alone kept in the light (Exp. 2, Table IV). There was

Table IV

		Comparative intensities of reddish colour				
			In coloured bottles in dark			
Exp.	Time of observation hr.	In light 2 mg. adr.	2 mg. adr.	2 mg. adr. +1 mg. asc.	2 mg. adr. +2 mg. asc.	2 mg. adr. +4 mg. asc.
1	3	Trace	–	–		
	24	+	–	–		
	48	++	–	+		
	72	++	–	++		
	96	++*	+	++*		
2	20	+	–	+	+	
	44	++	+	++	++	
3	24	Trace	–	–	–	–
	48	+	–	–	–	–
	72	++	–	+	+	+
	96	++	+	++	++	++*
	110	++*	++	++*	++*	++*

* Yellow tint. adr. = adrenaline hydrochloride. asc. = ascorbic acid.

no definite rule as regards the time of first appearance of colour in the solutions kept in the light. The rate at which the reddish colour appeared and increased in intensity in the different solutions is indicated by the results of 3 experiments recorded in Table IV.

It is clear that light accelerates the coloration of adrenaline solutions and that when the effect of this factor is eliminated the solutions containing adrenaline and ascorbic acid become coloured earlier than similar solutions containing no ascorbic acid. If the coloration of adrenaline solutions is accepted as an indication of oxidative destruction, this result would mean an acceleration of the destruction of adrenaline by ascorbic acid. When, however, the pressor activity of the solutions was actually tested, a contradictory result was obtained.

Solutions were prepared as before, some being made up one day before the others, for the purpose of studying the correlation between the intensity of colour and the pressor activity. The pressor activity of all the solutions was compared with that of a freshly prepared adrenaline hydrochloride solution buffered at *p*H 7 by measuring their effect on the blood pressure of one and the same pithed cat. Equal doses of 0·5 ml. were injected. The results of a number of such experiments are given in Table V.

Table V

Soln. no.	Contents of soln. mg. per 100 ml.	Condition	Age of preparation days	Intensity of colour	Mean rise in blood pressure mm. Hg	Strength relative to fresh soln. adr.
1	2 adr.	In light	3	+ +	28	0·25
2	2 adr.	In dark	3	+	40	0·36
3	2 adr.	In light	2	+	40	0·36
4	2 adr.	In dark	2	−	54	0·48
5	2 adr. + 1 asc.	In dark	2	+	54	0·48
6	2 adr. + 2 asc.	In dark	2	+	54	0·48
7	2 adr. + 4 asc.	In dark	2	+	54	0·48

adr. = adrenaline hydrochloride. asc. = ascorbic acid.

Observations obtained after allowing the solutions to stand more than 3 days are not recorded owing to complications which occurred after this period, such as the development of a yellow tint.

It can be seen that under the specified conditions of concentration and *p*H all solutions, whether coloured or not, were less active than a similar but freshly prepared adrenaline solution. Even the colourless solution (4) lost some of its pressor activity. According to Ball & Chen [1933] the first oxidation product, the *o*-quinone compound corresponding to adrenaline, is not coloured. This is supported by the work of Raper [1926; 1927] on the oxidation of the related substance dihydroxyphenylalanine. If we assume that in solution (4) a complete or partial transformation into the *o*-quinone compound did take place, it follows that autoxidation of the OH groups of the catechol ring results in the formation of a compound of lower activity than the original adrenaline. The comparison of (4) with (3) does not support the view that the *o*-quinone compound is quite devoid of activity.

In the solutions containing adrenaline alone at *p*H 7, the earlier production of colour in the light than in the dark was accompanied by greater diminution in pressor activity (3, 4). The further increase of colour intensity was accompanied by further loss of pressor activity (1, 2, 3). Although there was an earlier development of colour in the light than in the dark, when the intensity of coloration became the same in both cases the solutions had the same activity (2,3).

According to Green & Richter [1937], the red-coloured substance adrenochrome is produced by further oxidation of the primary *o*-quinone product of adrenaline oxidation and involves the removal of one hydrogen atom from the —NH— group of the side chain and one from the *o*-quinone ring, with the formation of an indole ring. According to them the first oxidation product is extremely unstable and at *p*H 7 undergoes an almost instantaneous change into the red-coloured adrenochrome which is physiologically inactive. The association of colour production with the formation of an indole ring from the *o*-quinone derivative of dihydroxyphenylalanine has also been pointed out by Raper [1926; 1927]. The red-coloured adrenochrome, therefore, is the second product of autoxidation of adrenaline although it constitutes the first visible stage in its oxidation. It is clear from the present work that this latter stage is photocatalysed, since there is an earlier development of colour in solutions of adrenaline alone kept in the light than in solutions prepared at the same time but kept in the dark, in which the original adrenaline proved to have undergone a change which is responsible for most of the total lowering of activity. This explains the further diminution in pressor activity as the colour develops (3, 4) and the proportionality of this diminution to the intensity of coloration (1, 2, 3, 4). It also explains why, when the intensity of coloration of solutions kept in the light and in the dark becomes the same, they have the same pressor activity, for the amount of active and inactive substances at this point will be the same in both cases.

Although there was an early development of colour in the solutions of adrenaline containing ascorbic acid, these showed an activity similar to that of a colourless solution prepared at the same time and kept under the same conditions, and a greater activity than a coloured solution also prepared at the same time but kept in the light. When the solutions 4–7 (Table V) were allowed to stand for another day, nos. 5–7 containing ascorbic acid developed maximum red colour and solution 4 developed a slight coloration; but while the pressor activity of nos. 5–7, when again compared with that of a freshly prepared solution, kept constant as before, that of no. 4 had diminished. These experiments show that ascorbic acid at *p*H 7 has a limited protective effect on the activity of adrenaline. This limited effect was still more prominent when 1 in 10^6 solutions of adrenaline alone and adrenaline containing the same amount of ascorbic acid were allowed to oxidize in the presence of air and light; it was found that after 24 hr. the solutions containing adrenaline alone completely lost their activity, while those containing ascorbic acid were still active, though they had a lower activity than freshly prepared solutions of the same concentration and reaction. The inactivation of solutions of adrenaline alone, in the light or in the dark, is proportional to the colour development, but no such proportionality exists in the solutions containing ascorbic acid as well. In the latter solutions, both the development of colour and the preservation of activity are independent of the amount of ascorbic acid present.

We can find no explanation of these experimental facts except by assuming (1) that the red-coloured product obtained with solutions of adrenaline containing ascorbic acid is different from the inactive adrenochrome produced in solutions of adrenaline alone; it might result from a reaction between the *o*-quinone primary oxidation product and ascorbic acid; its association with colour is an indication that it would also be an *o*-quinone; (2) that the protective effect of ascorbic acid hinders the transformation of the *o*-quinone primary oxidation product of adrenaline into the inactive adrenochrome. These assumptions explain (1) the preservation of an activity equal to that of the colourless

primary oxidation product by the solutions containing adrenaline and ascorbic acid, (2) the earlier appearance of coloration in these solutions than in those of adrenaline alone under the same conditions, (3) why further increase in colour intensity can take place without further loss of pressor activity, contrary to what occurs in solutions of adrenaline alone, since the increased intensity in the presence of ascorbic acid may simply mean the transformation of the colourless *o*-quinone into a coloured one of the same activity. According to Green & Richter [1937] ascorbic acid prevents the catalytic oxidation of adrenaline to adrenochrome.

Modified dichlorophenol-indophenol titrations showed that at *p*H 7 the rate of disappearance of the reduced form of ascorbic acid was rapid and was the same in solutions containing adrenaline as in similar concentrations of ascorbic acid alone. Complete disappearance took place in a few hours in both cases. The accelerated appearance of coloration in solutions containing adrenaline and ascorbic acid cannot therefore be due to the reduced form of ascorbic acid since the latter disappears long before the appearance of colour; the oxidation products of ascorbic acid may be responsible. These observations prove that the continued presence of the reduced form of ascorbic acid itself is not essential for the maintenance of the activity previously indicated to take place in the solutions of adrenaline with ascorbic acid, since it has been shown that the preservation of an activity equal to that of the primary oxidation product can continue after the development of the colour and, as can be seen, after the disappearance of the reduced form of ascorbic acid. Whether the coloured substance formed in the solutions which had contained ascorbic acid is concerned with the preservation of activity remains to be investigated.

The loss of potency in adrenaline solutions containing ascorbic acid (indicated in Table V) when compared with a freshly prepared solution of adrenaline alone was shown by further experiments to be demonstrable even before the complete disappearance of the reduced form of ascorbic acid, while at *p*H 7 a freshly prepared solution of adrenaline containing ascorbic acid had the same potency as a freshly prepared solution of adrenaline alone. It appears, therefore, that at *p*H 7 there is at first a simultaneous oxidation of the reduced form of ascorbic acid and of adrenaline which is changed to the corresponding *o*-quinone, thereafter there is a protection of the *o*-quinone compound from further oxidation.

These *in vitro* experiments were concerned with the protective effect of ascorbic acid on the pressor activity of adrenaline when the former was deprived of its acidic properties. In such experiments there is a rapid oxidation of ascorbic acid; a better protective effect on adrenaline might be expected in animal tissues, owing to their tendency to keep ascorbic acid in the reduced form.

The catalytic oxidation of adrenaline to the inactive adrenochrome was shown by Green & Richter [1937] to take place in the animal body by (1) a cyanide-insensitive system present in heart and skeletal muscle and (2) the cytochrome-indophenol oxidase present in all tissues. In the present work, as well as in the experiments of the above investigators, the hindrance of adrenochrome formation by ascorbic acid is indicated. In the intact animal, even if an increased concentration of ascorbic acid is effective in hindering adrenochrome formation, it is evident from the present work that the influence of this factor is combated by other factors, so that the same destruction of adrenaline is produced in normal animals as in those saturated with ascorbic acid. The possibility of adrenaline destruction in the body by mechanisms other than adrenochrome formation can be imagined from the different modes of adrenaline oxidation reported by previous investigators. Thus Weinstein & Manning [1937]

found a substance having the properties of protocatechuic acid in the urine of rabbits in which adrenaline had been injected, which indicates an oxidative removal of the side chain of the adrenaline molecule. Blaschko *et al.* [1937] have reported the presence of adrenaline oxidase in mammalian liver and other tissues; this was subsequently found by Richter [1937] to be an amine oxidase which induced an oxidative deamination in the side chain with the production of an aldehyde and methylamine.

It seems that any interference with the side chain of the adrenaline molecule by oxidative splitting or closure to form an indole ring results in complete loss of pressor activity, while autoxidation of the hydroxyl groups in the catechol ring simply lowers the activity.

SUMMARY

1. Small doses of unneutralized vitamin C injected into a pithed cat have no effect on the blood pressure, but large doses have a depressor effect. When the vitamin is adjusted to the physiological *p*H it has no action. The effect of the large doses of the unneutralized vitamin is ascribed to its acidity rather than to its real influence.

2. At the physiological *p*H, vitamin C augments neither the magnitude nor the duration of the adrenaline effect on the blood pressure, but the acidic vitamin diminishes both its magnitude and its duration.

3. The destruction of adrenaline is the same in normal intact animals as in those saturated with vitamin C. Any protective effect of the latter on adrenaline is combated by other destructive factors operating in the organism.

The parallelism previously observed between the rate of glycogenolysis caused by adrenaline and the degree of saturation of the body with vitamin C cannot be ascribed to a prolongation of the effect of the former through a protective effect of the latter.

4. *In vitro* at *p*H 7 ascorbic acid has a limited protective effect on the pressor activity of adrenaline. It appears that this protection consists in a temporary hindrance of further autoxidation of a primary oxidation product of adrenaline which has a lower pressor activity than the original compound. It is suggested that this primary product is the colourless *o*-quinone corresponding to adrenaline. The continued presence of the reduced form of ascorbic acid is not essential for this protection.

5. The fact that coloration occurs earlier in mixtures of ascorbic acid and adrenaline than in solutions of the latter alone cannot be taken as a sign of earlier destructive oxidation as in the case of adrenaline alone; it may be due to some sort of reaction between the primary oxidation product of adrenaline and the products of ascorbic acid oxidation. The coloured substance produced in these mixtures is suggested to be an *o*-quinone possessing a pressor activity equal to that of the colourless *o*-quinone compound.

6. The effect of light on the destruction of adrenaline appears to be concerned particularly with hastening the second step of autoxidation which is associated with the development of colour.

We wish to express our thanks to Prof. G. V. Anrep and Dr A. Hassan for the encouragement and the interest they have shown in this work.

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CLXXXIX. CARBOHYDRATES IN PROTEINS

I. THE CARBOHYDRATE COMPONENT OF CRYSTALLINE EGG ALBUMIN

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THE question of the occurrence of carbohydrate groups as essential parts of the structure of proteins (apart from mucins and mucoids) has been the subject of many investigations, none of which however has yielded an unequivocal result.

Before the peptide theory of proteins was generally accepted, numerous papers were published concerning the carbohydrate content of proteins. Pavy [1893] hydrolysed coagulated egg-white first with alkali and then with dilute acid and obtained glucosazone from the hydrolysate. He assumed that all proteins contain fairly large amounts of carbohydrate and that the preformed sugar in the protein molecule is responsible for the conversion of protein into carbohydrate which occurs in the animal body. These contentions were the subject of a heated controversy and, although Pavy's physiological hypotheses were somewhat bizarre, his chemical observations were correct. Eichholz [1898] confirmed Pavy's findings by showing that egg albumin, from which mucin and mucoid had been carefully removed, gave on hydrolysis a substance which reduced Fehling's solution and from which an osazone of M.P. 208° could be obtained. Later, when crystalline egg albumin had been prepared, Hofmeister [1898] and Langstein [1902] obtained an osazone from the hydrolysate of the crystalline protein and Seemann [1900] isolated glucosamine hydrochloride from a thrice crystallized sample. The amounts isolated, however, were very small and it was generally assumed that ordinary proteins are composed of amino-acids only and that the presence of sugars in hydrolysates is due to impurities such as mucoids (see Levene [1923] and Plimmer [1917]).

Much later, the question was taken up again by Fränkel & Jellinek [1927] who hydrolysed apparently non-crystalline egg albumin with baryta, removed amino-acids with neutral lead acetate and precipitated the polysaccharide with ammoniacal lead acetate. They repeated this separation several times and finally obtained, by precipitation with alcohol, an optically inactive polysaccharide. This substance on acid hydrolysis gave mannose and glucosamine. Levene & Mori [1929], following the work of Fränkel & Jellinek, introduced as an additional step in the purification of the polysaccharide the use of mercuric sulphate for the removal of nitrogenous impurities. These authors, however, obtained only small amounts of polysaccharide from repeatedly recrystallized egg albumin and concluded therefore that the carbohydrate is not derived from the egg albumin itself but from ovomucoid. Rimington [1929; 1931] applied the same method of hydrolysis and isolation to mixed serum proteins and also obtained mannose and glucosamine hydrochloride as ultimate hydrolysis products.

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Sørensen [1934], using a colorimetric technique, detected carbohydrates in nearly all proteins examined by her, and estimated that crystalline egg albumin contains 1·8 % of mannose.

There remains therefore considerable doubt as to whether crystalline proteins do in fact contain carbohydrates as part of their molecular structure. The positive colour reactions for carbohydrates which are given by highly purified albumin preparations are not sufficient to establish the point with certainty, whilst the failure of workers such as Levene & Mori to obtain more than traces of carbohydrate from purified proteins may well be due to defects in the methods employed.

Thus, prolonged hydrolysis with strong alkali as used by Levene & Mori is well known to be destructive of many carbohydrates and it will in fact be shown later in this paper that such treatment is harmful to the polysaccharide obtained from egg albumin. Moreover, it can hardly be claimed that the method used by these workers for the isolation of the carbohydrate, depending as it does on quantitative removal of amino-acids by neutral lead acetate and mercuric sulphate and selective precipitation of the polysaccharide by ammoniacal lead acetate, is well adapted to the separation of a small amount of carbohydrate in a pure state from a protein hydrolysate.

The object of the work to be described in the present paper has been first to develop a new method for the isolation of carbohydrates from proteins which should be free from the objections mentioned above and secondly to apply the new method to the study of what is perhaps the best-defined protein at present available, namely crystalline egg albumin.

Attempts to separate carbohydrate from egg albumin by physical means

The possibility of the separation of carbohydrate from crystalline proteins by physical means was first investigated, and for this purpose the carbohydrate content of crystalline egg albumin was estimated by the orcinol method after successive recrystallizations, after denaturation and after ultrafiltration. The justification for the use of a colorimetric method for the estimation of carbohydrate will appear later from the fact that the amount of sugar finally estimated by actual isolation agrees satisfactorily with the colorimetric value.

Table I. *Change of carbohydrate content with recrystallization*

Crystallization no.	% Carbohydrate
1	2·8
2	2·0
3	1·8
4	1·75
5	1·8
6	1·8
7	1·8

Table I shows that the carbohydrate content of crystalline egg albumin falls during the first two recrystallizations but is not affected by further repetitions of the process. The final value of 1·8 % carbohydrate calculated as mannose obtained agrees well with that recorded by Sørensen [1934]. Crystallization in itself therefore, so far from effecting a separation of carbohydrate from the protein, leads to a product of constant carbohydrate content.

An attempt was then made to separate the carbohydrate from the protein by ultrafiltration through filters of known porosity. Table II shows that a membrane of average pore diameter 5·5 mμ, which retains egg albumin completely

[Elford & Ferry, 1936], is also impermeable to the carbohydrate associated with it; moreover, for membranes of intermediate porosity the ratios of carbohydrate and nitrogen in the ultrafiltrates are constant within the limits of experimental error. It is clear therefore that the carbohydrate is associated with particles of molecular size similar to that of egg albumin.

Table II. *Ultrafiltration of egg albumin*

Protein	Average pore diameter of membrane in mμ	Carbohydrate content		Protein content		Carbohydrate content of protein	
		Supernat.	Ultrafiltr.	Supernat.	Ultrafiltr.	Supernat.	Ultrafiltr.
		mg. per ml.		mg. per ml.		%	
Native	35	0·324	0·321	1·786	1·714	1·80	1·86
,,	11	0·424	0·095	2·370	0·452	1·77	2·10
,,	9	0·464	0·043	2·740	0·224	1·70	2·00
,,	5·5	0·545	0·010	3·260	0·003	1·70	—
Acid denat.	15	0·325	0·010	1·820	—	1·77	—
Heat denat.	15	0·039	0·060	0·207	0·010	1·88	—

Finally Table II also shows that no carbohydrate is split off on denaturation with acid. The fact that the carbohydrate, which passes readily through the 15mμ membrane when combined with the native protein, is completely retained with the protein after the latter has been denatured is convincing evidence that the carbohydrate must be associated with a denaturable protein and not, for instance, with contaminating mucin. This is confirmed by the fact that no carbohydrate is liberated during denaturation by heat.

The impossibility of separating carbohydrate from recrystallized egg albumin by any of the physical processes described affords reasonable ground for the assumption that the carbohydrate forms an integral part of the protein molecule. With respect to denaturation the possibility remains that the carbohydrate group may play some part in the intramolecular mechanism of this process while remaining attached to the protein molecule.

New method for the isolation of carbohydrate from protein hydrolysates

From the experiments of Rimington [1931], it can be deduced that trypsin does not hydrolyse the polysaccharide derived from serum proteins. It is reasonable to expect that the carbohydrate in egg albumin will be similarly resistant and hydrolysis by trypsin was therefore chosen for the present work. Introduction of extraneous carbohydrate with the trypsin was minimized by keeping the amount of enzyme as low as possible; since the (colorimetric) carbohydrate content of the trypsin preparation used was very small, the amount of carbohydrate introduced with the enzyme was actually less than 0·2% of the total.

The new method of isolation of the carbohydrate from the hydrolysis mixture was based on the following considerations. Ketene reacts quantitatively with α-amino-acids (and α-imino-acids) [Neuberger, 1938] in aqueous solutions of neutral and alkaline reaction to form *N*-acetyl compounds [Bergmann & Stern, 1930]. In the basic amino-acids the amino groups only are acetylated; thus the action of ketene on histidine and arginine yields α-*N*-acetylhistidine and α-*N*-acetylarginine respectively; these acetylated acids retain a zwitterion structure, and are therefore insoluble in organic solvents. Aliphatic hydroxyl groups on the other hand are not acetylated by ketene in aqueous solution, as shown by the fact that ketene reacts with glucosamine to form *N*-acetylglucosamine only

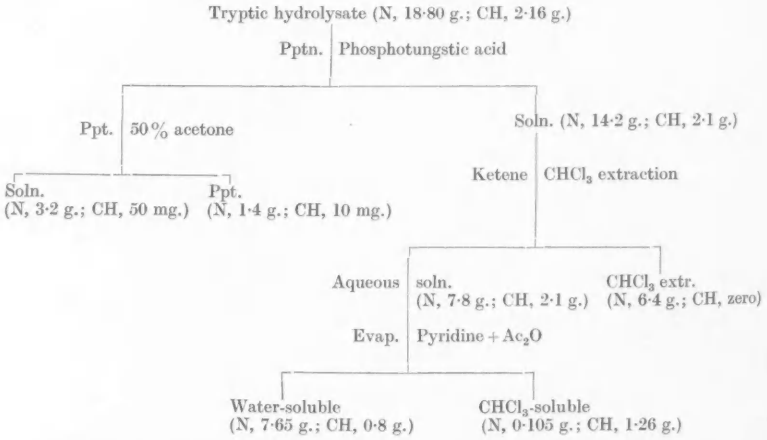
[Bergmann & Stern, 1928]. It is to be expected therefore that if a mixture of amino-acids and carbohydrate is treated with ketene, all amino-acids except histidine and arginine will be converted into compounds which are soluble in organic solvents, whereas carbohydrates will remain unchanged, apart from acetylation of the amino group of glucosamine or other amino-sugars.[1]

By treatment of a tryptic hydrolysate of protein with ketene and subsequent exhaustive extraction of the acidified solution with chloroform, after removal of basic amino-acids with phosphotungstic acid, it should therefore be possible to remove a large proportion of nitrogenous material without affecting the carbohydrate.

Now carbohydrates in general are readily and completely acetylated by acetic anhydride and pyridine; treatment of the material remaining in the chloroform-extracted solution with these reagents should therefore convert the carbohydrate into a chloroform-soluble condition in which it can easily be separated from the remaining nitrogenous contaminants. It should then be possible to deacetylate the chloroform-soluble acetylated carbohydrate under mild conditions, and to isolate the free carbohydrate by precipitation with alcohol.

A difficulty might arise in the application of the procedure just outlined owing to the formation of chloroform-soluble acetaminoketones by the reaction studied by Dakin & West [1928]; actually it has been shown [Neuberger, 1938] that if the temperature of the acetic anhydride-pyridine mixture is kept low such formation of acetaminoketones from amino-acids is almost entirely suppressed in favour of the formation of acetamino-acids; in any case, even if small amounts of acetaminoketones and/or acetamino-acids should pass into the final chloroform extract, these will be unaffected by the deacetylation process and, being readily soluble in alcohol, will remain in the mother liquor when the free carbohydrate is precipitated.

Table III. *Distribution of nitrogen (N) and carbohydrate (CH) during process of fractionation*



[1] The differential extraction of acetamino-acids by organic solvents as a means of separating amino-acids is being examined by Mr R. Ll. M. Synge in the Biochemical Laboratory, Cambridge.

The procedure finally adopted for the isolation of the polysaccharide has been as follows. The hydrolysate is first treated with phosphotungstic acid to remove basic amino-acids and incompletely digested protein. The material not precipitated by phosphotungstic acid is then treated with ketene, and acetylated amino-acids are removed so far as possible by chloroform extraction. The material obtained on evaporating the aqueous solution is treated with pyridine and acetic anhydride at 0°. The acetylated polysaccharide thus obtained is then extracted from the aqueous solution of the reaction product by chloroform and carefully deacetylated by dilute alkali in the cold, and the free carbohydrate is precipitated by addition of alcohol.

Table III shows the distribution of nitrogen and carbohydrate in the different fractions in a typical experiment; it will be seen that very little carbohydrate is lost by precipitation with phosphotungstic acid and no carbohydrate at all passes into the first chloroform extract. It is clear from Table III that the partition coefficients of some acetamino-acids between chloroform and water are not very favourable for their complete removal from the aqueous phase.

It also appears from Table III that only 60 % of the polysaccharide is obtained in the second chloroform extract after one treatment with pyridine and acetic anhydride. In later experiments it was found that 95 % of the total carbohydrate could be obtained in the final alcohol precipitate by repeating the acetylation process on the residue.

Properties of the carbohydrate

General. The substance obtained, having the appearance and general properties of a polysaccharide, was homogeneous, since samples obtained by fractional precipitation with alcohol had identical rotations, molecular weights and nitrogen and carbohydrate contents (Table IV). Colour reactions indicated

Table IV. *Physical and chemical properties of different preparations and fractions obtained by fractional precipitation with alcohol*

	Nitrogen content %	$[\alpha]_D$	Mol. wt. uncorr. for ash	Carbohydrate content %
Preparation 1	4·85	+21·5	1150	47·0
Preparation 2	4·95	+22·0	—	48·0
Preparation 3	5·05	+22·0	—	50·0
Preparation 4	4·95	+22·5	1130	48·0
Fraction I precipitated by 50 % alcohol	4·85	+22·0	—	46·5
Fraction II precipitated by 65 % alcohol	4·90	+20·5	1160	50·1
Fraction III precipitated by 75 % alcohol	5·10	+22·5	1110	49·0
Fraction IV precipitated by 90 % alcohol	5·15	+22·0	—	—

the presence of hexose and acetamino-sugar, whilst the presence of significant amounts of ketoses, pentoses and uronic acids could be excluded. The substance gives the ninhydrin reaction after acid hydrolysis; this does not necessarily imply the presence of amino-acids however since glucosamine hydrochloride gives a typical ninhydrin test. The negative biuret test showed that the polysaccharide was not contaminated by higher peptides. Negative colour reactions further indicated the absence of tyrosine, tryptophan, histidine, arginine and cystine from the preparation.

Component sugars. The polysaccharide itself had no free reducing group, but reducing sugars were liberated on acid hydrolysis. Fig. 1 shows that the maximum reducing value on hydrolysis with 1·5*N* HCl at 100° is obtained after about 5 hr.

If correction is made for the "salt error" and the slightly higher reducing equivalents of mannose and glucosamine as compared with glucose, the amount of reducing sugar in the polysaccharide can be calculated to be about 75%. On the other hand the amount of sugar other than amino-sugar, as determined colorimetrically, was about 48%, a figure nearly identical with that obtained for the fermentable sugar. Thus, about 50% of the polysaccharide or 68% of the reducing sugars is present as fermentable hexose. On the other hand direct colorimetric estimation of amino-sugar indicated a content of 25% calculated as glucosamine [Elson & Morgan, 1933]. Thus, it can be concluded that the reducing sugar obtained on hydrolysis consists of two-thirds of a fermentable hexose and one-third of an amino-sugar.

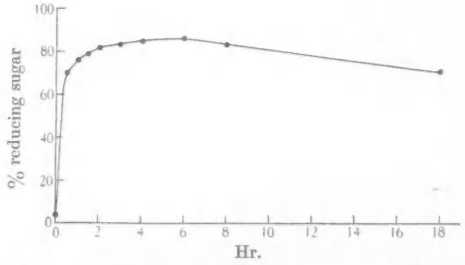


Fig. 1. Liberation of reducing sugar on hydrolysis of polysaccharide with 1·5 *N* acid at 100°.

Mannose was isolated from a hydrolysate of the polysaccharide in the form of the phenylhydrazone and the *p*-bromophenylhydrazone. The amounts corresponded to 36 and 38% of the polysaccharide, whilst in model experiments under the conditions used for the isolation of the hydrazones, mannose could be recovered to the extent of 75–80%. If such a correction for the incomplete recovery of mannose is applied to the values obtained, the content of mannose in the polysaccharide falls between 45 and 48% which is in satisfactory agreement with the values obtained from the fermentable reducing sugar content and from colorimetric determination.

Glucosamine was isolated by a newly devised method as 2:4-dihydroxybenzylideneglucosamine. The amounts isolated in different experiments corresponded only to about 60% of the value found colorimetrically. The presence of an amino-sugar other than glucosamine can therefore not be excluded with complete certainty.

Nitrogenous components other than amino-sugars. The maximum content of amino-sugar (calculated as glucosamine) accounts for no more than 40% of the total nitrogen of the polysaccharide; on the other hand, after acid hydrolysis under conditions which would not destroy amino-sugars, 60–70% of the total nitrogen appears as amino-nitrogen (Van Slyke) and about 20% as ammonia.

In addition to amino-sugar(s) there must therefore be present in the polysaccharide a nitrogenous compound which yields its nitrogen partly as amino-nitrogen and partly as ammonia on acid or alkaline hydrolysis, the proportion of ammonia being probably larger in the latter case (see next paragraph). This compound has not so far been identified; colour reactions and consideration of its properties seem to exclude all known amino-acids and its instability both towards acid and alkali make its closer investigation difficult. It is hoped that

its further study may become possible if success is attained in the search for an enzyme which will hydrolyse the polysaccharide.

Action of alkali. Fig. 2 shows that 50 % of the total nitrogen is liberated from the polysaccharide as ammonia by *N* NaOH at 100° after 16 hr. The fact that the yield of mannose as phenylhydrazone after a subsequent acid hydrolysis was greatly diminished indicated that the sugar had been partially destroyed by the alkaline treatment. Similarly, the fact that only 60 % of the remaining nitrogen or 30 % of the original nitrogen was present as amino-nitrogen showed that some

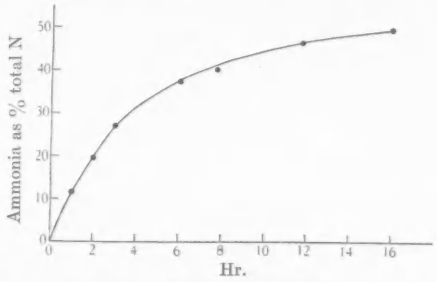


Fig. 2. Rate of liberation of ammonia on hydrolysis of polysaccharide with *N* NaOH at 100°.

glucosamine also had been destroyed. This apparent instability of glucosamine bound in a polysaccharide towards strong alkali is in contrast to the behaviour of methyl glucosaminide which is very stable under the conditions used [Moggridge & Neuberger, 1938].

After alkaline hydrolysis the polysaccharide gives a positive test with Ehrlich's reagent which is not easy to explain, since *N*-acetyl methyl glucosaminide and *N*-acetyl phenyl glucosaminide, when heated with alkali under the same conditions, do not give this reaction. A possible explanation is that a part at least of the glucosamine is bound to the unknown nitrogenous substance by a linkage more unstable to alkali than the *O*-glucoside linkage. On alkaline treatment, this linkage might be split more quickly than the *N*-acyl linkage, and the compound thus partly deaminated and partly condensed to a ring system giving a positive test with Ehrlich's reagent.

Acetyl content. Acetyl estimations showed that the polysaccharide contained 10·65 % acetyl groups, presumably in the form of *N*-acetyl. The amount of acetyl corresponds nearly exactly to the amount of amino-nitrogen obtained after acid hydrolysis, and it may be concluded that the acetyl groups are linked partly to glucosamine and partly to the unknown nitrogenous component. Since the isolation of the polysaccharide involves acetylation it is impossible to say whether the glucosamine in its original combination in the protein is *N*-acylated or not.

Content of the polysaccharide in egg albumin. As will be shown later, 3·5 g. of the polysaccharide complex are obtained from 100 g. of egg albumin. If the yield of the polysaccharide is assumed to be 95 %, this corresponds to a value of 3·65 g. per 100 g. of protein. Since the mannose content of the polysaccharide complex is 50 %, a value of 1·8 g. of mannose per 100 g. of protein is obtained which is in perfect agreement with the colorimetric value. From the analytical figures therefore egg albumin with mol. wt. 40,000 would contain 4 mol. of mannose and 2 mol. of glucosamine.

Estimations of the mol. wt. of the polysaccharide complex indicated a value of 1250, which, on the basis of mol. wt. 40,000 for egg albumin, corresponds to a polysaccharide content of the protein of 3·15%. The agreement with the value obtained from isolation and colorimetric methods is sufficiently good to justify the assumption that four mannose and two glucosamine residues are linked up as one polysaccharide chain with the protein to form one large prosthetic group.

Experimental

Preparation of egg albumin

Egg albumin was crystallized according to Kekwick & Cannan [1936]. Samples from each crystallization were dialysed against frequent changes of distilled water for 10 days and nitrogen and carbohydrate were estimated. For the estimation of carbohydrate the method of Sørensen & Haugaard [1933] was used employing mannose as standard. The nitrogen content of egg albumin was assumed to be 15·65%.

Effect of heat-denaturation on carbohydrate content

Five times recrystallized egg albumin (20 g.) was denatured by heating in aqueous solution at 80° for 20 min. and the suspension was dialysed against frequent changes of distilled water at 0°. The combined dialysates were evaporated to dryness *in vacuo*. A minute amount of solid matter giving a very weak Molisch reaction was obtained. It was impossible to obtain from it an osazone, even after acid hydrolysis, nor could a phenylhydrazone or phenylhydrazide be prepared; the presence of hexoses and hexonic acids was thus excluded.

Ultrafiltration experiments

The membranes were made and standardized by Dr Elford and the technique used was that described by Elford & Ferry [1934]. Nitrogen and carbohydrate were estimated in samples of the supernatant fluid and of the ultrafiltrate. Heat-denatured protein was prepared by keeping an electrolyte-free solution of the protein at 85° for 20 min. The suspension was centrifuged at 2000 r.p.m. for 5 min. and the supernatant fluid was filtered through a 1·5μ membrane which allowed about 12% of the protein originally present to pass through. Acid-denatured protein was prepared by keeping a protein solution for 10 hr. at *p*H 1 at a temperature of 30° and then adjusting the *p*H to the isoelectric point. Protein thus denatured passes readily through a 1·5μ membrane, but is completely retained by a 15mμ membrane.

Isolation of the polysaccharide from egg albumin

Egg albumin, five times recrystallized and nearly completely freed from sodium sulphate by dialysis, was used in all experiments. A 5% solution of the protein was denatured by heating and the reaction of the suspension was adjusted to *p*H 7·8. Trypsin (Merck) was then added in an amount corresponding to 0·3% of the dry weight of the protein. Alkali was added frequently to adjust the *p*H of the digest to 7·8 and further quantities of trypsin were added about twice a week. The enzymic hydrolysis was followed by alkalimetric titration in 90% alcohol according to Willstätter & Waldschmidt-Leitz [1921], correction being made for the alkali added; the digestion was continued for 4–6 weeks. The total amount of trypsin added varied between 0·8 and 1·1 g. per 100 g. of protein.

The working up of this digestion mixture may be described in detail for a typical experiment.

The enzymic hydrolysate of 120 g. of egg albumin was acidified to *p*H 1 with HCl. Phosphotungstic acid (150 g.) in 0·1 *N* HCl was added and the mixture was left for 3 days in the cold room. The precipitate was filtered off and washed with a 10 % solution of phosphotungstic acid in 0·1 *N* HCl. The precipitate was suspended in warm 50 % acetone in which most of it dissolved and the suspension was extracted five times with an amyl alcohol-ether mixture. The aqueous suspension was filtered and the solution was analysed for carbohydrate and nitrogen (fraction I). The precipitate was again suspended in water and dissolved by addition of NaOH (fraction II). The phosphotungstic acid was removed by $Ba(OH)_2$ and the excess $Ba(OH)_2$ precipitated by H_2SO_4.

The filtrate from the phosphotungstic acid precipitation was freed from excess phosphotungstic acid by amyl alcohol-ether extraction until a sample gave no precipitation with $Ba(OH)_2$. The solution was then neutralized with 2 *N* NaOH and concentrated to low bulk.

To the neutralized solution sufficient NaOH was then added to adjust the reaction to *p*H 9 and ketene was passed in until the ninhydrin reaction became negative; this took about 18 hr. Small additions of alkali were made at intervals to maintain the *p*H at about 6. The solution was then concentrated at low pressure and acidified to *p*H 1·6 with HCl. Acetylated amino-acids were now removed by continuous extraction with chloroform in the apparatus shown in Fig. 3. The extraction was generally stopped after 12 hr. since it was found that continuance for a further 50 hr. did not appreciably increase the amount of nitrogen in the chloroform layer. The chloroform solution was evaporated to dryness, the residue was taken up in water and nitrogen and carbohydrate were estimated.

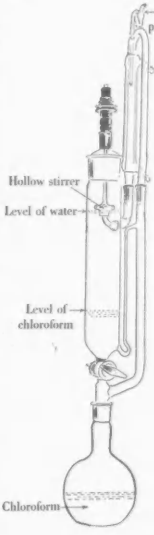


Fig. 3.[1] Apparatus for continuous extraction of an aqueous solution with chloroform.

The aqueous solution was concentrated *in vacuo* and dried in a desiccator first over conc. H_2SO_4 and finally over P_2O_5 to a thick syrup. To this syrup were added pyridine (250 ml.) and acetic anhydride (350 ml.), and the mixture was shaken mechanically for 2 days at 2°. It was then poured into ice-water and left for 5 hr. The solution was concentrated under very low pressure, the residue diluted with water and the latter again evaporated. The aqueous solution of the residue was then made acid to Congo red and extracted repeatedly with chloroform, the combined chloroform extracts being washed with water containing NaCl and dried. After removal of the chloroform an oil was obtained which was dissolved in alcohol. Sufficient lithium hydroxide was added to keep the solution alkaline to phenolphthalein for at least half an hour. After 2 hr. more in the ice-chest the alkali was exactly neutralized with the required amount of dilute HCl and the solution concentrated under reduced pressure nearly to dryness; the residue was dissolved in a small amount of water and alcohol was added to a final concentration of 95–97 %. The precipitate was centrifuged off

[1] This apparatus can be purchased from Messrs Quickfit and Quartz, Ltd.

after standing for several hours in the ice-chest, washed several times with ice-cold absolute alcohol and dried over P_2O_5.

The mother liquor from the alcohol precipitation was evaporated and a further small amount of precipitate was obtained on addition of alcohol. Altogether 2·9 g. of material were obtained.

The aqueous solution obtained from the last chloroform extraction and containing unacetylated polysaccharide was evaporated to dryness, and subjected to reacetylation followed by further treatment as above; 1·2 g. more of material were obtained, identical in all respects with that derived from the first acetylation. A third acetylation gave no further product.

General properties of the polysaccharide

The substance obtained by the method described is very slightly pigmented and fairly hygroscopic; it is very soluble in water, somewhat soluble in aqueous alcohol and insoluble in absolute alcohol and acetone. Its aqueous solution has *p*H 5·5–6·0. The nitrogen contents of different samples as estimated by the Kjeldahl method varied between 4·85 and 5·1 %. (It was found that reliable nitrogen values for glucosamine or glucosamine-containing compounds could only be obtained by the ordinary Kjeldahl method if ashing with H_2SO_4 were continued for about 6 hr.; with SeO_2 as catalyst however half an hour sufficed.)

The polysaccharide gives no biuret reaction even in a 20 % solution; it gives a ninhydrin reaction only after acid hydrolysis. The Millon and Sakaguchi reactions are negative both with the untreated polysaccharide and with samples hydrolysed with 2 *N* HCl and with conc. HCl. In view of the claim of Rimington [1931] that histidine is combined with the polysaccharide obtained from serum proteins the Pauly reaction was carried out on samples hydrolysed under many different conditions: 5 mg. samples were hydrolysed with 2 *N* HCl at 100°, conc. HCl at 125° and with 10 % $Ba(OH)_2$ at 100° all for 5 hr. In all cases only a slight yellow-orange colour was obtained with diazobenzenesulphonic acid in sodium carbonate solution, which was very different from the colours given by histidine and tyrosine. The Hopkins-Cole and Rosenheim tests for tryptophan were negative before and after alkaline hydrolysis. Tests for sulphur and phosphorus were likewise negative.

The Molisch reaction was positive up to a dilution of 1 : 1,000,000. The polysaccharide itself did not reduce Fehling's solution, but became strongly reducing on mild acid hydrolysis. A strongly positive test for *N*-acetylglucosamine [Morgan & Elson, 1934] was obtained after hydrolysis with 0·01 *N* HCl at 100° for 1 hr. The naphthoresorcinol, phloroglucinol and Seliwanoff tests were all negative on samples hydrolysed under difficult conditions. Carbohydrate estimations by the method of Sørensen & Haugaard gave values of 47–50 % using mannose as a standard (see Table IV).

Estimation of the molecular weight

The molecular weight was estimated by an isopiestic method worked out by Dr G. S. Hartley similar to that described by Robinson & Sinclair [1934]. A rectangular silver plate containing three circular grooves was placed in a bulb-shaped glass apparatus which could be exhausted. Polysaccharide solutions of different concentrations were placed in the outside grooves while a solution of KCl of known strength was put into the middle groove. The apparatus was carefully exhausted on an oil pump and left for 2 days at 25°; it was then opened and the amounts of solutions in the three grooves were determined by weighing.

Osmotic equilibrium was reached, and the concentrations of polysaccharide in the two outside grooves were identical.

The method may be described in detail for one experiment. 0·0896 g. of a solution containing 0·00706 g. of polysaccharide was placed in groove 1, 0·05840 g. of a solution containing 0·00132 of polysaccharide in groove 3, whilst in groove 2 0·09168 g. of 0·05 *N* KCl was introduced. At the end of the experiment grooves 1 and 3 contained 0·0928 and 0·0178 g. of solution respectively indicating concentrations of polysaccharide of 7·45 and 7·61 %. The values show that osmotic equilibrium between the two polysaccharide solutions had in fact been established, and it can be assumed that equilibrium had also been reached between the polysaccharide and the KCl solutions. In groove 3 containing KCl, the amount of solution had increased to 0·117 g. indicating that the two polysaccharide solutions are in equilibrium with a 0·0409 *N* solution of KCl. Using an osmotic coefficient for KCl of 0·943 and recalculating the concentrations of polysaccharide in g. per 1000 g. of solvent, values for the mol. wt. of 1120 and 1150 respectively were obtained.

Estimation of ash content

10·62 mg. of the polysaccharide were ashed by heating at 500° for 6 hr. The residue amounted to 0·0192 mg. corresponding to an ash content of 0·1735 %. If it is assumed that the ash consists only of lithium chloride a correction for the value of the mol. wt. can be made. The values of the mol. wt. corrected on such a basis are 1245 and 1275.

The homogeneity of the polysaccharide

450 mg. of the polysaccharide were dissolved in 1 ml. of water and 1 ml. of alcohol was added. The precipitate was centrifuged off and dried. To the supernatant solution pure alcohol was added and the precipitate formed again centrifuged off. By thus increasing the alcohol concentration several fractions were obtained. Optical rotations, mol. wt. and nitrogen and carbohydrate contents of the different fractions were estimated; the results are recorded in Table IV.

Estimation of reducing sugars

66·6 mg. of the polysaccharide were dissolved in 11 ml. of 1·5 *N* HCl and heated at 100° using a reflux condenser. 1 ml. samples were taken out at intervals, neutralized to 99 % with NaOH and made up to 2·5 ml. The reducing values were estimated according to Hanes's [1929] modification of the Hagedorn-Jensen method. The results in terms of glucose are recorded in Fig. 1. The reducing value rises rapidly on hydrolysis until it reaches a maximum at about 6 hr.; thereafter it decreases slowly, presumably owing to destruction of the sugar. The maximum reducing value expressed as glucose corresponds to a content of 85 % of reducing sugar in the polysaccharide. This value is too high for two reasons. Mannose and glucosamine which occur in the polysaccharide have higher reducing equivalents than glucose; expressed in terms of glucose the molecular reducing equivalents of mannose and glucosamine were found by the Hagedorn-Jensen method to be 101 and 103·5 % respectively. Secondly, it was noted that if an amount of sodium chloride is added to a glucosamine solution similar to that formed on neutralizing the acid hydrolysate, the apparent reducing value of glucosamine is increased by about 10–12 % (see Moggridge & Neuberger, [1938]). If correction was made for these two factors a value of about 75 % of reducing sugar in the polysaccharide was obtained.

Estimation of fermentable sugar

30·2 mg. of polysaccharide were dissolved in 6 ml. of 1·5*N* HCl and heated in a sealed tube at 100° for 6 hr. The solution was then neutralized and made up to 20 ml. One 2 ml. sample of this solution was incubated with washed baker's yeast at 37° for 20 min. according to the method of Somogyi [1929]. The yeast was centrifuged off and the reducing value of the supernatant solution estimated. In a second sample the reducing value was estimated without previous treatment with yeast. Controls with yeast alone were carried out at the same time. The amount of sugar expressed as glucose in the sample not treated with yeast was 2·5 mg., whilst in the sample treated with yeast it amounted only to 0·81 mg. after correction for the reducing value of yeast itself had been made.

Estimation of amino-nitrogen

The polysaccharide itself does not give off any nitrogen in the Van Slyke apparatus. On hydrolysis the following amounts of amino-nitrogen are found:

	%
After 5 hr. hydrolysis with 1·5*N* HCl	3·2
After 8 hr. hydrolysis with 2·5*N* HCl	3·6
After 8 hr. hydrolysis with 5*N* HCl	3·2

These values were obtained on shaking for 5 min. In one experiment—after hydrolysis with 5*N* HCl—shaking was continued for 30 min. and the value was increased to 4·2 %. The apparent increase on prolonged shaking is probably due to the ammonia formed on acid hydrolysis. It may be noted that glucosamine hydrochloride gives off all its nitrogen on shaking for 5 min.

Acetyl estimation

Acetyl groups were determined according to Pregl & Soltys [1929]. In two experiments values of 10·61 and 10·67 % of acetyl were obtained.

Estimation of amino-sugar

Amino-sugar was estimated by Sørensen's [1938] modification of the method of Elson & Morgan [1933]. A standardization curve was obtained with solutions of glucosamine hydrochloride; the colorimetric readings were done on a Zeiss step-photometer. The mean value from several experiments corresponded to a content of 29·1 % of amino-sugar expressed as glucosamine hydrochloride or 24·3 % expressed as glucosamine.

Isolation of mannose

(*a*) *Isolation as phenylhydrazone.* 99 mg. of polysaccharide were dissolved in 1 ml. 1·5*N* HCl and heated in a sealed tube at 100° for 6 hr. The neutralized solution was filtered and made up to 3 ml. To this solution 0·2 ml. of glacial acetic acid and 0·3 ml. of freshly distilled phenylhydrazine were added. After a short time a crystalline precipitate formed which was filtered off after standing for 4 hr., washed with two lots of 0·5 ml. of 15 % acetic acid and dried. It amounted to 51 mg. and had M.P. after two recrystallizations 189° (uncorr.); a sample mixed with authentic mannose phenylhydrazone showed no depression of M.P. (Found: N (Dumas), 10·1 %. $C_{12}H_{18}O_5N_2$ requires N, 10·33 %.)

(*b*) *Isolation as* p-*bromophenylhydrazone.* 100 mg. of polysaccharide were dissolved in 10 ml. 1·5*N* HCl and heated at 100° for 5½ hr. The solution was evaporated to dryness at reduced pressure, the temperature of the bath not

exceeding 36°. The residue was taken up in water, neutralized and treated with 150 mg. *p*-bromophenylhydrazine in 50% acetic acid. Crystallization started at once; after 4 hr. standing the precipitate was filtered off, washed with 25% alcohol and dried. It amounted to 71 mg.

The twice recrystallized hydrazone had M.P. 202° (uncorr.); a sample mixed with authentic mannose *p*-bromophenylhydrazone showed no depression of M.P. No osazone could be obtained from the residual solution.

Isolation of glucosamine

Difficulties were encountered in attempts to separate glucosamine from hydrolysates of the polysaccharide until a new derivative of glucosamine was found which is very suitable for purposes of isolation. Glucosamine condenses at slightly alkaline reaction with 2:4-dihydroxybenzaldehyde to form a Schiff's base which crystallizes readily and which, in contrast with other Schiff's bases of glucosamine, is stable towards excess alkali. The reaction is almost quantitative as shown by the following experiments.

Preparation of 2:4-dihydroxybenzylidene-l-glucosamine. 215 mg. glucosamine hydrochloride (1 mol.) and 150 mg. $KHCO_3$ (1·5 mol.) were dissolved in 2·5 ml. water. 175 mg. 2:4-dihydroxybenzaldehyde (1·25 mol.) dissolved in 2 ml. 40% ethyl alcohol were added to the solution. Crystallization started after 15 min.; the mixture was left overnight in the ice-chest and the yellow crystals were then filtered off and washed with 2 ml. 40% alcohol. Yield: 265 mg. or 90% of the theoretical. The yield is not decreased by the presence of other sugars.

The substance was recrystallized from aqueous alcohol. It is soluble in alcohol and hot acetone, insoluble in water and ether. The substance has M.P. 116° (uncorr.) and decomposes at 118°. (Found: N (Kjeldahl), 4·54%. $C_{13}H_{17}O_7N$ requires N, 4·67%.) The substance mutarotates; the initial $[\alpha]_D = 312°$ (in alcohol); after 42 hr. $[\alpha]_D = 228°$.

If the substance is recrystallized by adding a large amount of water to its concentrated alcoholic solution and leaving the mixture overnight a new compound is formed which is pale yellow in colour and has M.P. 142°. Analysis indicates that the elements of water have been added to the Schiff's base. The same change occurs if the non-hydrated form is kept for some time exposed to the air. (Found: C, 49·15; H, 6·05%. $C_{13}H_{17}O_7N$ requires C, 49·27; H, 6·04%.)

The rotation of the new compound is slightly different from that of the Schiff's base; $[\alpha]_D$ (initial) = 299° (in alcohol), after 42 hr. $[\alpha]_D = 198°$.

Formation of a hydrochloride. 2:4-Dihydroxybenzylideneglucosamine forms with hydrochloric acid a hydrochloride of the hydrated compound containing 1 equiv. HCl. 280 mg. of the Schiff's base were dissolved in hot acetone and 0·25 ml. 5*N* HCl was added. A white crystalline precipitate formed at once which was filtered off and dried. It amounted to 320 mg. The substance is easily soluble in water and slightly soluble in alcohol, insoluble in ether and acetone. (Found: N, 3·8; Cl, 9·8%. $C_{13}H_{20}O_8NCl$ requires N, 3·96; Cl, 10·04%.)

An alcoholic solution treated with an equivalent amount of aqueous acid gives an identical product.

As found by Dimroth [1902] 2:4-dihydroxybenzylideneaniline is able to add on the elements of water and to form a hydrochloride. By analogy with his interpretation, the reactions here described may be represented as follows:

$$C_6H_{11}O_5.NH_2 + HOC.C_6H_5O_2 \rightarrow C_6H_{11}O_5.N{:}CH.C_6H_5O_2 + H_2O$$

$$\rightarrow C_6H_{11}O_5.NH.CH(OH).C_6H_5O_2 \xrightarrow{+HCl} C_6H_{11}O_5.NH_2^+.CH(OH).C_6H_5O_2 + Cl^-.$$

Recovery of glucosamine. Although the 2:4-dihydroxybenzylidene compound is quite stable to acid in the cold, it is split quantitatively on heating in acid solution.

150 mg. of dihydroxybenzylidene glucosamine were dissolved in 5 ml. 65% alcohol containing 1·5*N* HCl and heated under reflux on a water bath for 30 min. The crystalline precipitate which formed on cooling was increased by the addition of ether. The precipitate was filtered off and dried. The product was identified by nitrogen content and rotation as glucosamine hydrochloride, and the yield was 90% of the theoretical.

Isolation of glucosamine from the polysaccharide. 500 mg. of polysaccharide were hydrolysed with 5 ml. 5*N* HCl for 3 hr. at 100°. The solution was filtered and evaporated nearly to dryness under reduced pressure; the residue was taken up in water and the solution was again filtered. 500 mg. of phosphotungstic acid dissolved in 2·5 ml. 0·1*N* HCl were now added and the mixture was left overnight. The precipitate was centrifuged off and washed twice with, altogether, 2·5 ml. of a 5% phosphotungstic acid solution in 0·1*N* HCl. The combined solution and washings were extracted with amyl alcohol-ether and the aqueous solution was concentrated to low bulk, again filtered and allowed to evaporate to dryness in a desiccator. The residue which consisted of large plates admixed with amorphous material, was taken up in a small quantity of water, heated with alcohol and again evaporated to remove the last traces of water. Absolute alcohol was now added and the crystalline material was filtered off. It amounted to 120 mg.

The crystalline material was dissolved in 3 ml. 30% alcohol to which $KHCO_3$ (80 mg.) and 2:4-dihydroxybenzaldehyde (100 mg.) dissolved in 0·5 ml. 30% alcohol were added. After 6 hr. in the ice-chest the crystals were filtered off and amounted to 80 mg. The material was dissolved in alcohol and filtered; the solution was evaporated nearly to dryness and the residue recrystallized by addition of water. The substance had M.P. 142°; a sample mixed with the hydrated form of 2:4-dihydroxybenzylideneglucosamine showed M.P. 141°. It agreed with the latter also in optical rotation, having $[\alpha]_D$ (initially) = 304° (in alcohol), after 39 hr. $[\alpha]_D = 204°$. (Found: C, 49·20; H, 6·01%. $C_{13}H_{19}O_8N$ requires C, 49·27; H, 6·04%.)

Isolation of glucosamine hydrochloride. 35 mg. of the Schiff's base were dissolved in 2 ml. 1·5*N* HCl in 65% alcohol and the solution heated for 30 min.; the crystalline material was filtered off, washed with anhydrous ether and dried. Yield: 22·8 mg. (Found: C, 32·75; H, 6·51; N, 6·38%. $C_6H_{14}O_5NCl$ requires C, 33·4; H, 6·54; N, 6·50%.) In experiments involving different conditions of hydrolysis no better yields of the dihydroxybenzylidene compound could be obtained.

Nitrogen precipitated by phosphotungstic acid after acid hydrolysis

The phosphotungstic acid precipitate was decomposed with hydrochloric acid and amyl alcohol-ether and the aqueous solution was made up to known volume. A nitrogen estimation showed that 28% of the total nitrogen of the polysaccharide was precipitable by phosphotungstic acid after acid hydrolysis. The polysaccharide itself is not precipitated by phosphotungstic acid. The Pauly reaction was negative and no reaction for pyridine (chlorodinitrobenzene) could be obtained in the solution recovered from the phosphotungstate. Ammonia estimations by the method of Conway & Byrne [1933] indicated that about 55% of the nitrogen contained in the precipitate consisted of ammonia. (The ammonia was identified by analysis in the form of ammonium chloride.) Colorimetric

tests with Nessler's reagent indicated that the ammonia is not formed by the alkali used in its estimation. The residual nitrogen contained in the precipitate could not be accounted for; all attempts to prepare derivatives (chloroplatinate, chloroaurate, picrate, picrolonate, reineckate) led only to amorphous gums.

If milder conditions were used for acid hydrolysis the amount of ammonia present in the phosphotungstic acid precipitation was smaller, but it was still impossible to obtain crystalline products from this fraction.

Alkaline hydrolysis

Only small amounts of volatile base are evolved on boiling the polysaccharide with dilute alkali; e.g. 1·4 or 2·8 % of the total nitrogen were obtained as volatile base on boiling the polysaccharide for 1 hr. with 0·01 *N* and 0·1 *N* NaOH respectively, but much larger amounts are obtained on boiling with *N* NaOH.

51·1 mg. of polysaccharide were dissolved in 8 ml. *N* NaOH and the solution was boiled under reflux, the vapours being trapped in a receiver containing a known amount of 0·01 *N* HCl. The acid solution was back-titrated against 0·01 *N* NaOH at suitable intervals, and the results are recorded in Fig. 2. The experiment was stopped after 16 hr. In another experiment with 500 mg. polysaccharide the combined solutions containing the volatile base were made alkaline and distilled in the micro-Kjeldahl apparatus into a receiver containing HCl. The distillate was evaporated to dryness and the crystalline residue dried. The total yield was 48 mg. 10·3 mg. were used for nitrogen estimation. (Found: N, 26·05 %. NH_4Cl requires N, 26·16 %.)

The alkaline solution of the polysaccharide was acidified and hydrolysed for 5 hr. with 1·5 *N* HCl. Only about 100 mg. of mannose phenylhydrazone could be obtained, a yield of 14 % as against 37 % obtained from non-alkali-treated polysaccharide.

In another experiment in which 500 mg. of the polysaccharide were hydrolysed with 10 % $Ba(OH)_2$ at 100° for 7 hr. 37 % of the total nitrogen was given off as ammonia. Ba^{++} was removed exactly from the alkaline solution by H_2SO_4 and $BaSO_4$ was filtered off. The solution of the polysaccharide after alkaline hydrolysis gave a strong reaction with Ehrlich's reagent. To the solution were now added 5 ml. of Hopkins's reagent ($HgSO_4$ in 5 % H_2SO_4) and the mixture was left for 2 days. The mercuric sulphate precipitate was decomposed by H_2S and $Ba(OH)_2$ and the solution was evaporated nearly to dryness under reduced pressure. No colour reaction for tryptophan could be obtained nor was it possible to prepare a crystalline derivative from this fraction.

Discussion

It is felt that the work described in this paper places beyond all doubt the question of the occurrence of carbohydrate in crystalline egg albumin. The constancy of carbohydrate content on repeated crystallization and the consistent failure of all attempts at physical separation of protein and carbohydrate fractions, even by processes such as denaturation which must involve intramolecular change, leave no escape from the conclusion that a constant amount of carbohydrate is firmly attached to the protein molecule.

The second conclusion which emerges from the present work concerns the nature of the carbohydrate group, since for the first time a method has been devised for the isolation of this group in a condition which may be assumed to be essentially unaltered. Viewing the properties of the complex isolated from egg albumin in the light of this assumption we find that it consists of

a polysaccharide of molecular weight about 1200, made up for the most part of mannose and glucosamine in the proportion of two molecules of the former to one of the latter, but containing also an unidentified nitrogenous constituent. In all probability the glucosamine occurs in the actual protein-carbohydrate complex in the acetylated condition as it does in other natural products containing glucosamine, such as chitin, but this point cannot be decided by the method at present available; nor can the possibility of the presence of an amino-sugar other than glucosamine in the egg albumin polysaccharide be completely excluded, although it seems extremely remote.

Unfortunately the methods employed in the present work are incapable of giving us any definite information concerning the mode of combination of the polysaccharide with the rest of the protein molecule, except in so far as the liberation of the polysaccharide by tryptic hydrolysis suggests the participation of a peptide linkage; it is possible that the unidentified nitrogenous constituent of the polysaccharide may play some part in the combination and that a knowledge of the nature of this constituent might enable us to assign a definite role to the polysaccharide in the structure of the natural protein. A more exact analysis of the situation must await the development of new methods of attack and further speculation at the present time serves no useful purpose; it is even now however possible to state with some certainty, on the basis of the findings recorded above, that the polysaccharide forms a single prosthetic group with one point of attachment to the molecule of egg albumin, the word prosthetic implying no more than that the group in question is made up of compounds other than amino-acids.

One more point arises which may be briefly considered here. The discovery in recent years of the predominant part which is played by carbohydrate groups in the determination of the specificity (as distinct from antigenic capacity) of many bacterial antigens naturally gives rise to the speculation that a similar state of affairs may exist among the proteins in so far as the latter contain carbohydrate groups. Hitherto it has been impossible to put this question to the test since no method has been available whereby the carbohydrate group could be separated from a protein without subjection to treatment calculated to alter its biological properties entirely. With the aid of the gentle method of isolation described in the present paper it seems that the problem can be tackled with more hope of success, and experiments are in fact already in hand to examine how far, if at all, the immunological specificity of crystalline egg albumin is determined by the carbohydrate group which it contains.

Summary

1. Previous work on the occurrence of carbohydrate in proteins other than mucins and mucoids is briefly reviewed.

2. It is shown that the carbohydrate content of crystalline egg albumin becomes constant on recrystallization and that it is impossible to separate the carbohydrate from the protein by denaturation or ultrafiltration.

3. A method for the isolation of the carbohydrate group in almost quantitative yield from egg albumin is described which is based on tryptic hydrolysis and involves no chemical treatment more drastic than acetylation and deacetylation under the mildest conditions.

4. The properties of the polysaccharide thus isolated are described in detail. It is shown to have mol. wt. about 1200 and to be composed almost certainly of four molecules of mannose and two of glucosamine, together with an unidentified nitrogenous constituent. The complex is regarded as forming a single

prosthetic group with one point of attachment to the molecule of natural egg albumin.

5. The preparation and properties are described of 2:4-dihydroxybenzylidenеglucosamine, a compound useful for the isolation and characterization of glucosamine.

6. The possible significance of the carbohydrate group in relation to the structure and immunological properties of egg albumin is briefly discussed.

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CXC. NOTE ON THE ACTION OF ACETYLATING AGENTS ON AMINO-ACIDS

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In connexion with the development of a new method for the isolation of carbohydrate from protein hydrolysates [Neuberger, 1938] it became necessary to study certain aspects of the reactions of amino-acids and related compounds with pyridine-acetic anhydride and ketene.

Reactions of amino-acids with pyridine and acetic anhydride

If amino-acids are treated in glacial acetic acid solution with the theoretical amount of acetic anhydride, optically active acetamino-acids are obtained, while if a large excess of acetic anhydride is used, racemization and/or formation of azlactones takes place [Bergmann *et al.* 1926; Bergmann & Zervas, 1928]. Excess of acetic anhydride causes racemization even in aqueous solution in the presence of alkali [du Vigneaud & Meyer, 1932]. If the reaction with acetic anhydride, however, is carried out at 100° in the presence of pyridine, CO_2 is split off and acetaminoketones result [Dakin & West, 1928]. It has been found by the present author that this anomalous reaction is suppressed at low temperature. Thus two typical amino-acids, histidine and phenylalanine, which by treatment with pyridine and acetic anhydride at 100° are almost quantitatively converted into the corresponding acetaminoketones, give, when the reaction is carried out at 2°, very good yields of acetamino-acids; only in one case was there an indication that a minute trace of a ketone was formed. It seems, however, that at least partial racemization of some amino-acids is induced by this method of acetylation also.

Action of ketene on amino-acids and related compounds

Bergmann & Stern [1930] found that the alkali salts of simple amino-acids such as leucine and phenylalanine, or in some cases the amino-acids themselves, react with ketene to form *N*-acetylated compounds whilst tyrosine in presence of an excess of alkali gives *ON*-diacetyltyrosine. This method of acetylation which is quantitative and does not lead to side-reactions has been used to mask amino- and phenolic hydroxyl-groups in proteins [Herriott & Northrop, 1934; Stern & White, 1938] and it has been tacitly assumed that changes in the protein molecule, under the action of ketene, are in fact restricted to these two types of groups. The experiments of Bergmann & Stern [1930] were confined to racemic monoaminomonocarboxylic acids, *l*(−)-tyrosine and *l*(+)-glutamic acid and the question of racemization was not studied in detail by these authors; in the present work the study of the reaction has been extended to more complex amino-acids, in which cases, as will appear, partial or complete racemization may occur.

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As might be expected, the α-imino-group of proline is readily acetylated by ketene in alkaline solution. Basic amino-acids on the other hand react only with their amino-groups; thus from *l*(+)-arginine and *l*(−)-histidine the corresponding mono-acetyl compounds were obtained. Lysine, however, reacts with ketene to form a diacetyl derivative. It may be noted in passing that although the glyoxaline group of histidine does not react with ketene in aqueous solution, glyoxaline and 4-(or 5)-methylglyoxaline when dissolved in indifferent solvents are changed by ketene into non-crystalline unsaturated compounds not giving the Pauly reaction. Probably ring fission and simultaneous acetylation occur and the acetylated unsaturated diamines [Bamberger & Berlé, 1893] formed in the first instance, undergo polymerization.

Aliphatic hydroxyl groups do not react readily with ketene even in indifferent solvents as shown by the fact that from ethyl *dl*-β-hydroxyglutamate in ether the *N*-acetyl compound only was obtained. In marked contrast to this is the behaviour of aliphatic thiol groups. If cysteine dissolved in 2 equiv. of alkali is treated with ketene both the amino- and thiol-groups are acetylated; α-thiolpropionic acid is likewise acetylated in aqueous alkali.

However, if α-thiolpropionic acid is treated with ketene in ether instead of water, a mixed anhydride of S-acetyl-α-thiolpropionic and acetic acids is formed. The mixed anhydride which can be distilled decomposes in the presence of water to form S-acetyl-α-thiolpropionic acid; this reaction is not surprising since ketene is known to form mixed anhydrides with carboxylic acids in indifferent solvents [Hurd & Dull, 1932]. Dicarboxylic acids, however, react with ketene in indifferent solvents to form internal anhydrides; thus from β-phenylglutaric acid the corresponding anhydride was readily produced.

It was noticed in several cases, especially when an excess of alkali was used, that the action of ketene on amino-acids resulted in partial or complete racemization. This is probably due to the action of acetic anhydride, which is almost certainly the first reaction product between ketene and water, and which, as mentioned above, racemizes acetamino-acids at alkaline reactions. Thus arginine is completely racemized, in agreement with the observation of du Vigneaud & Meyer [1932] who found that *N*-acetyl-*l*(+)-arginine is racemized by acetic anhydride even without addition of alkali.

From these results it can be concluded that if proteins are treated with ketene in alkaline solution acetylation may take place at amino-, thiol- and phenolic-groups, whilst guanidino- and aliphatic hydroxyl-groups will remain unaffected. The possibility that the glyoxaline group might react with ketene, when the carboxyl group is blocked, cannot be completely excluded; it is well known that the Bamberger fission does not take place with substituted glyoxalines containing free carboxyl groups in the side chain whilst the esters of such compounds undergo the fission in the normal manner [Windaus *et al.* 1921]; it is therefore possible that, although the glyoxaline ring of free histidine is not attacked by ketene in aqueous solution, the amino-acid may be so attacked when it is in peptide linkage.

Experimental

Action of pyridine and acetic anhydride on amino-acids in the cold

Preparation of acetyl-dl-*phenylalanine.* *dl*-Phenylalanine (1 g.) was suspended in a mixture of pyridine (5 ml.) and acetic anhydride (8 ml.) and the suspension was shaken mechanically for 2 hr. at 2°. The clear solution was left for 2 days at this temperature and then poured into ice-water. After standing for 4 hr. the solution was evaporated to dryness under reduced pressure,

the evaporation being twice repeated after addition of small amounts of water. The residue was taken up in a small amount of ethyl acetate, the ethyl acetate solution was dried and the substance was crystallized by addition of light petroleum. Yield was 85 % of the theoretical. Recrystallization was effected from water. At no stage of the preparation could a positive nitroprusside reaction for ketones be obtained. M.P. 148° (uncorr.). (Found: N (Kjeldahl), 6·85 %. $C_{11}H_{13}O_3N$ requires N, 6·76 %.) Titration: 20·9 mg. neutralized 5·10 ml. 0·02 *N* NaOH (cresol red). $C_{11}H_{13}O_3$, titrating as a monocarboxylic acid, requires 5·05 ml.

Preparation of acetylhistidine. *l*(−)-Histidine (1 g.) was suspended in pyridine (5 ml.) and acetic anhydride (8 ml.) and the mixture was shaken at 2° for 2 hr. and then left for 2 days at this temperature. Since part of the solid still remained undissolved the suspension was shaken for 6 hr. more at room temperature. The solution, which now contained only a small amount of solid matter, was poured into ice-water. A very faint reaction with nitroprusside was obtained. Removal of pyridine and acetic acid was carried out as described above. The residue obtained after evaporation was placed in a dish in a desiccator over absolute alcohol at a pressure of 60 mm. After a few hours the product crystallized out and was filtered off and dried. Yield 80 % of the theoretical. Recrystallization was effected by addition of acetone to a concentrated aqueous solution. (Found: N (Kjeldahl using SeO_2 as catalyst), 19·35 %. $C_8H_{13}O_4N_3$ requires N, 19·53 %.) M.P. 155° (not sharp) $[\alpha]_D = +22·5°$. Acetyl-*l*(−)-histidine [Bergmann & Zervas, 1928] has M.P. 169° and $[\alpha]_D = +44·7°$. It is clear therefore that partial racemization has occurred during acetylation.

Action of ketene on amino-acids and related compounds

Ketene was generated from acetone as described by Herriott [1934] in an all-glass apparatus in order to avoid the formation of polymerization products.

*Preparation of acetyl-*l(−)*-proline.* *l*(−)-Proline (250 mg.) was disssolved in 25 ml. 0·4 *N* NaOH and treated with ketene for 45 min. 5 ml. 2 *N* HCl were then added and the solution was evaporated to dryness under reduced pressure. The residue was taken up in alcohol, the solution filtered and again evaporated. The residue was taken up in ethyl acetate and crystallized by addition of light petroleum and recrystallized from chloroform and light petroleum. Yield was 80 % of the theoretical. M.P. 115° (uncorr.). (Found: N, 8·84 %. $C_7H_{12}O_3N$ requires 8·86 %. Titration: 12·6 mg. neutralised 4·01 ml. 0·02 *N* NaOH. $C_7H_{12}O_3N$ requires 3·98 ml.) $[\alpha]_D = -104°$. *N*-acetyl-*l*(−)-proline as described by du Vigneaud & Meyer [1932] has M.P. 117° (uncorr.) and $[\alpha]_D = -106·7°$.

*Preparation of acetyl-*dl*-arginine.* 1 g. of crystalline arginine prepared [Bergmann & Zervas, 1926] from the hydrochloride of $[\alpha]_D = +17°$, was dissolved in 25 ml. CO_2-free water and ketene was passed into the solution for 2 hr. The solution was evaporated to dryness and the residue was dried over KOH in a desiccator. The syrup was dissolved in a small quantity of water and crystallized by addition of alcohol. The product had M.P. 253° (decomp.) and lost no water on drying over P_2O_2 at 60° at 1 mm. (Found: N, 26·03 %. $C_8H_{11}O_3N_4$ requires 25·93 %.) A 5 % solution was optically inactive.

An inactive preparation of the acetyl compound was also obtained when a solution of *l*(+)-arginine hydrochloride ($[\alpha]_D + 17·0°$) containing 2 equiv. of NaOH was treated with ketene at 0°.

Preparation of acetylhistidine. *l*(−)-Histidine, crystallized according to Vickery & Leavenworth [1927], was treated with ketene and the resulting

solution was worked up as described for arginine. The product was only partly racemized. It had $[\alpha]_D = +28{\cdot}5°$; pure acetyl *l*(−)-histidine has $[\alpha]_D = +40{\cdot}5°$.

Action of ketene on lysine. No crystalline acetyl derivative was obtained from lysine, but on treating 1 g. of *l*(+)-lysine dihydrochloride dissolved in water containing 3 equiv. of alkali with ketene a product was obtained which was soluble in alcohol and ethyl acetate. A solution, after ketene treatment, containing 5·7 mg. of total nitrogen per ml. gave 0·31 mg. of amino-nitrogen, in the Van Slyke apparatus, after shaking for half an hour, indicating that almost quantitative acetylation of both amino-groups had taken place.

Action of ketene on glyoxalines. Ketene was passed into a solution of 1 g. glyoxaline in anhydrous ether (20 ml.) which was kept at −15° to avoid formation of polymerization products of ketene. An amorphous product was obtained which was easily soluble in water, slightly soluble in alcohol and insoluble in ether and did not give a reaction with diazobenzenesulphonic acid in Na_2CO_3 even after acid hydrolysis. A chloroform solution of the substance took up bromine and decolorized alkaline permanganate. Boiling the product with methyl alcoholic HCl [Windaus *et al.* 1921] led to an amorphous product which did not take up bromine any more and from which only a partly crystalline *p*-nitrophenylosazone was obtained; the latter could not be further purified. The product obtained by the action of ketene took up hydrogen in the presence of Pd but only to the extent of 25 % of the amount calculated for an ethylenediamine.

Similar products were obtained by the action of ketene on 4(or 5)-methylglyoxaline.

Preparation of ethyl N-*acetyl*-dl-*β-hydroxyglutamate.* Ethyl *dl*-*β*-hydroxyglutamate hydrochloride (2·2 g.) was dissolved in a small quantity of water; to the chilled solution ether and solid K_2CO_3 were added until the aqueous layer formed a thick paste. The paste was extracted several times with ether and the combined extracts were dried first with K_2CO_3 and then with freshly prepared BaO. Into the ethereal solution ketene was passed at −15° for 1 hr. After removing the ketene by a stream of nitrogen the ethereal solution was concentrated to a syrup, which crystallized after standing in a desiccator at 0° for 2 days. Recrystallization was effected from ether. Yield was 53 % of the theoretical. M.P. 46° (uncorr.). (Found: N, 5·29 %. $C_{11}H_{19}O_6$ requires N, 5·32 %.) Acetyl content: found 17·1 %. $C_{11}H_{19}O_6$ requires N, 16·47 %.

Reaction of ketene with thiol groups

Preparation of NS-*diacetylcysteine.* *l*(+)-Cysteine hydrochloride (1 g.) was dissolved in a small quantity of water containing 3 equiv. of NaOH; ketene was passed into the solution for 1 hr. The solution was then acidified with 3 equiv. of H_2SO_4 and extracted with ethyl acetate; the residue from the evaporation of the combined and dried extracts was dissolved in a small quantity of ethyl acetate and, on addition of light petroleum (B.P. 120–140°), a crystalline precipitate was obtained. Yield 50 % of the theoretical. M.P. 111–112° (uncorr.). (Found: N, 6·84 %. $C_7H_{11}O_4NS$ requires 6·83 %.) Titration: 20·3 mg. neutralized 4·75 ml. 0·02*N* NaOH. $C_7H_{11}O_4NS$, as a monobasic acid, requires 4·95 ml. Acetyl estimation: acetyl found 41·3 %; calculated for 2 acetyl groups 41·95 %.

The substance is soluble in water, alcohol and most organic solvents. The nitroprusside reaction develops very slowly but the colour remains constant for many hours indicating a slow splitting of the thioester linkage by the ammonia employed in the reaction.

Preparation of S-*acetyl-α-thiolpropionic acid.* To a solution of freshly distilled α-thiolpropionic acid (5 g.) in freshly boiled out water (50 ml.) 30 ml. of 5*N* NaOH were added with exclusion of air. Ketene was passed into this solution for 2 hr. at 0°. The bulk of the acetic acid was then removed by extraction with ether; the solution was acidified with 30 ml. of 5*N* HCl and again extracted with ether. After removal of the ether an oil was obtained which could be distilled under very low pressure. It had B.P. 132–133° at 1 mm.; it was soluble in water, alcohol, ether and chloroform. (Found: S (Carius), 21·95 %. $C_5H_8O_3S$ requires S, 21·62 %.) The oil titrates as a monobasic acid; 142·4 mg. neutralized 9·69 ml. 0·1*N* NaOH (cresol red); calc. for $C_5H_8O_3S$, 9·62 ml. Saponification: 10 ml. 0·1*N* NaOH were added to the neutralized solution, and the mixture heated at 70° for 3 hr.; 1 ml. 0·1*N* HCl was then added and the solution was boiled to remove CO_2 and titrated with 0·1*N* NaOH to *p*H 7·5. Total amount of alkali required 9·65 ml.; calculated for 1 mol. of acid liberated 9·62 ml.

Preparation of a mixed anhydride of S-*acetyl-α-thiolpropionic and acetic acids.* 5 g. of α-thiolpropionic acid (freshly distilled) were dissolved in absolute ether (25 ml.) and treated with ketene. The solution was then evaporated to dryness and the oily residue which had a very pungent odour was dried in a desiccator over KOH and conc. H_2SO_4. It was finally obtained as a water-clear mobile liquid by distillation at 0·25 mm. with a bath-temperature of 77–83°. Yield was 5·59 g. (Found: S, 17·1 %. $C_7H_{18}O_4S$ requires S, 16·84 %.) The oil is soluble in most organic solvents, and insoluble in water; on prolonged contact with water, however, it dissolves and loses its pungent odour.

Preparation of S-*acetyl-α-thiolpropionic acid from the mixed anhydride.* 2·5 g. of the mixed anhydride were shaken with 5 ml. of water for 5 hr. when the oil had completely dissolved; the solution was then evaporated to dryness and the residue was left in a desiccator over KOH. It was then distilled; B.P. 133°/1 mm. (Found: S, 22·05 %. $C_7H_8O_3S$ requires S, 21·62 %.) Titration: 123·2 mg. required 8·36 ml. 0·1*N* NaOH for neutralization to cresol red; calculated for $C_5H_8O_3S$ as a monobasic acid 8·37 ml.

Preparation of β-phenylglutaric acid anhydride. β-Phenylglutaric acid (2 g.) dissolved in absolute ether (25 ml.) was treated with ketene. The oil obtained crystallized after 2 days' standing in a desiccator, and was recrystallized from benzene. Yield 77 % of the theoretical. M.P. 101°; a mixture with a sample prepared by the action of acetyl chloride showed no depression of M.P. (Found: C, 69·64; H, 5·35 %. $C_{11}H_{10}O_3$ requires C, 69·47; H, 5·30 %.)

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CXCI. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA

VIII. A NOTE ON THE EFFECT OF ANTICOAGULANTS

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Sperry & Schoenheimer [1935] concluded that for the determination of blood cholesterol, oxalated plasma was unsuitable. In one case they estimated the cholesterol contents of serum and oxalated plasma and found 409·5 and 300 mg. per 100 ml. respectively. They then instituted further comparisons and though they did not report finding any other differences of this degree, they stated that "the average difference between the amounts of total cholesterol in heparinized and oxalated plasmas was 15·3 % of the former". They also quoted the work of Schmidt [1935] who found that the corresponding differences in the amount of phospholipid as estimated in heparinized and oxalated plasma averaged 10 % of the former.

There can be little doubt that the chief explanation of these differences is the effect of the oxalate addition in producing some shrinking of the red corpuscles; the fluid so lost diluted the plasma. This effect was noted by Gram & Norgaard [1923], and Eisenman *et al.* [1925] estimated this shrinkage by determining the serum protein after the defibrination of the blood with and without oxalate addition. They calculated the relative change in serum volume from the oxygen capacity and cell volume on the one hand, and from the protein content on the other. Both these methods demonstrated that the addition of oxalate to the blood caused a relative increase of plasma volume averaging 6 %. Eisenman [1927] showed that the addition of neutral potassium oxalate to the extent of 0·17–0·2 % of the whole blood caused a reduction of cell volume averaging 2 vol. % of the whole blood, which corresponds to a dilution of the plasma averaging 3·5 %. She also showed that with higher concentrations of oxalate, up to 0·45 %, there might be a shrinkage of the corpuscles up to 7·4 vol. % of the whole blood, equivalent to a plasma dilution of about 12 %.

Wintrobe [1931–2], in a series of haematological studies, stated that heparin is the ideal anticoagulant as it does not affect the size of the red corpuscles, but that it cannot always be depended upon unless the extremely expensive purified material is employed. He noted that there was a shrinkage of 8·2 % of the corpuscular volume in oxalated blood as compared with heparinized blood, and consequently he used the factor 1·09 to obtain the real corpuscular volume from the value obtained by using oxalated blood. Now the average corpuscular volume is 42 % of the whole blood, so that a shrinkage of 8·2 % of the volume would represent the addition of 3·44 vol. of fluid to 58 vol. of plasma, i.e. there would be a dilution of the plasma by approximately 6 %. It is interesting to note that this figure is the same as that determined by Eisenman *et al.* [1925].

Wintrobe's estimations of the change of corpuscular and plasma volumes by relatively simple physical methods are likely to be more accurate than the estimation of either cholesterol or phospholipid in the plasma by any method. As Sperry & Schoenheimer's results showed differences more than twice as large as the probable degree of plasma dilution, we thought that it would be of interest to make a further series of determinations.

Table I. *Comparison of the cholesterol content of heparinized and oxalated plasmas*

Series	Cholesterol, mg. per 100 g. plasma: Free	Ester	Total	% of total as ester	Mean values: Free	Ester	Total	
D 1:								
Heparin	47	135	182	74·2	—	—	—	
	45	134	179	74·9	46	134·5	180·5	
% difference	**4·3**	**0·7**	**1·7**	—	**10·9**	**1·1**	**3·6**	% diminution produced by oxalate
Oxalate	42	132	174	75·8	—	—	—	
	40	134	174	77·0	41	133	174	
% difference	**4·9**	**1·5**	**0**	—	—	—	—	
D 2:								
Heparin	34	118	152	77·6	—	—	—	
	34	118	152	77·6	34	118	152	
% difference	**0**	**0**	**0**	—	**4·4**	**3·0**	**3·3**	% diminution produced by oxalate
Oxalate	32	115	147	78·2	—	—	—	
	33	114	147	77·6	32·5	114·5	147	
% difference	**3·1**	**0·9**	**0**	—	—	—	—	
D 3:								
Heparin	32	120	152	78·8	—	—	—	
	33	116	149	78·1	32·5	118	151·5	
% difference	**3·0**	**3·3**	**2·0**	—	**4·6**	**1·7**	**3·0**	% diminution produced by oxalate
Oxalate	31	119	150	79·6	—	—	—	
	31	113	144	78·6	31	116	147	
% difference	**0**	**5·0**	**4·0**	—	—	—	—	
H 1:								
Heparin	71	183	254	72·1	—	—	—	
	72	180	252	71·3	71·5	181·5	253	
% difference	**1·4**	**1·6**	**0·8**	—	**3·5**	**5·5**	**5·3**	% diminution produced by oxalate
Oxalate	68	170	238	71·4	—	—	—	
	70	173	243	71·3	69	171·5	239·5	
% difference	**2·9**	**1·8**	**2·0**	—	—	—	—	
*H 2:								
Heparin	70	187	257	72·9	—	—	—	
	69	186	255	73·0	69·5	186·5	256	
% difference	**1·4**	**0·5**	**0·8**	—	**2·9**	**5·4**	**4·7**	% diminution produced by oxalate
Oxalate	68	176	244	72·6	—	—	—	
	67	177	244	72·2	67·5	176·5	244	
% difference	**1·5**	**0·6**	**0**	—	—	—	—	

* These results are expressed as mg. cholesterol per 100 ml. plasma.

Cholesterol was determined in heparinized and oxalated plasmas by the method described by us [Gardner *et al.* 1938]. Blood was taken from normal individuals or from patients who were unlikely to have any disturbance of their

cholesterol metabolism. Each specimen was divided into two portions of which one was treated with neutral potassium oxalate to the extent of 0·2 g. per 100 ml., and the other with heparin (Hyson, Westcott and Dunning's preparation) to the extent of 1 mg. per 5 ml. of blood. In all experiments, duplicate determinations were made. Cholesterol is expressed in mg. per 100 g. of plasma except in one series (H 2) where determinations were made on 10 ml. portions of plasma, and hence the results are expressed as mg. cholesterol per 100 ml. plasma.

The average diminution of total cholesterol content produced by oxalating plasma was 3·8%, whereas the average difference between duplicate determinations of total cholesterol was only 1·1%. As we do not yet understand the significance of much greater variations from the average normal, this reasonably constant error of 4% is not a very grave matter.

Summary

1. Addition of neutral potassium oxalate to blood leads to a small shrinkage of the erythrocytes, with a consequent slight dilution of the plasma.

2. In cholesterol estimations on heparinized and oxalated plasma there was found to be a reduction of concentration produced by neutral potassium oxalate of about 4%. This figure is of the same order as the degree of shrinkage of the red cells.

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CXCII. CHANGES IN THE LIVERS OF MICE AFTER ADMINISTRATION OF 3:4:5:6-DIBENZCARBAZOLE

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The known carcinogenic hydrocarbons produce primary tumours at the site of application [cf. Cook *et al.* 1937] and one of them at any rate (1:2:5:6-dibenzanthracene) increases the incidence of lung cancer in those strains of mice in which this condition occurs spontaneously [Lynch, 1935; Andervont, 1935]. The feeding [Sasaki & Yoshida, 1935] or injection [Shear, 1937] of 4′-amino-2:3′-azotoluene induces malignant changes in the livers of rats and mice. This compound, however, does not have any marked carcinogenic action at the site of administration. 3:4:5:6-Dibenzcarbazole produces sarcomata as a result of subcutaneous injection into rats and epitheliomata and bile duct hypertrophy resembling cholangiomata when it is painted twice weekly on the skin of mice [Boyland & Brues, 1937]. Strong *et al.* [1938] found that the injection of 3:4:5:6-dibenzcarbazole into mice of the C.B.A. strain, in which hepatoma occurs spontaneously, increased the incidence of these tumours.

As 3:4:5:6-dibenzcarbazole produces bile duct proliferation and neoplastic changes in the livers an attempt has been made to correlate the histological appearances with certain chemical changes. This compound is much more toxic to mice than carcinogenic hydrocarbons such as 1:2:5:6-dibenzanthracene or methylcholanthrene.

Methods. Mixed stock mice previously fed on Purina Fox Chow for a week received a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole dissolved in olive oil. Mice weighing less than 16 g. were not used as it was found that the compound was more toxic to young mice. During the experiment the diet consisted entirely of Fox Chow. At varying intervals after the injection mice were weighed, killed and their livers removed. A small portion of the liver was taken for histological section, and the remainder was investigated chemically. In some cases a small piece was also taken for estimation of the glycolysis and respiration in the Warburg apparatus.

The liver tissue, after weighing, was ground in a centrifuge tube with 3 vol. of 5 % trichloroacetic acid, the suspension centrifuged and an aliquot portion of the supernatant fluid removed for determination of ascorbic acid and glutathione as described by Mawson [1935]; the ascorbic acid was first titrated with phenolindo-2:6:dichlorophenol and then the glutathione with iodine. The trichloroacetic acid precipitate was saponified with KOH on a boiling water bath and the "fat" estimated by the Liebermann method as described by Leathes & Raper [1925]. According to Dulière and Minne [1937] cholesterol is completely removed from blood serum by precipitation with trichloroacetic acid and we have found that estimations of the fat content on the trichloroacetic acid precipitate of liver give the same values as estimations on fresh tissue. The cholesterol in the

"fat" was determined by a modification of the method of Schoenheimer & Sperry [1934] for blood; the material was dissolved in 5 ml. acetone-alcohol (1:1) and 2 ml. 0·5 % digitonin in 50 % alcohol were added. The solution was allowed to stand overnight and next day the precipitate was centrifuged and washed once with acetone-ether (1 : 2) and twice with ether. The last traces of ether were removed in an oven at 40° and the precipitate dissolved in 4 ml. glacial acetic acid. 8 ml. acetic anhydride and 0·4 ml. conc. H_2SO_4 were added and the colour which developed was compared in a colorimeter with that of a cholesterol standard, using a red screen.

Results

Series 1. In the first experiment 0·5 mg. 3:4:5:6-dibenzcarbazole was given to each mouse, and there was a considerable mortality during the week following the injection. Of a series of 38 mice, 10 were alive on the 3rd day after injection; 4 of these were killed and only 3 of the remaining 6 were alive on the 5th day. Many of the livers were very pale and appeared fatty. The most striking chemical change was in the glutathione (Fig. 1); the amount in many cases was definitely

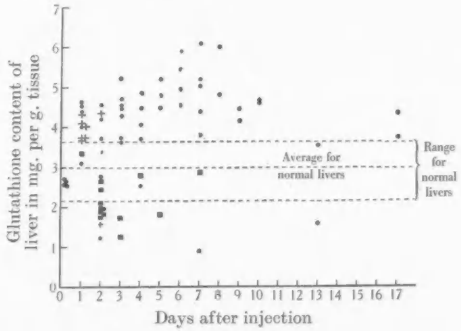

Fig. 1. The glutathione content of livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole. • Represents liver of normal histological appearance. ■ Represents liver with advanced necrosis. + Represents liver with early necrosis.

increased and in 3 mice was twice the normal average. This average (2·91 mg. per g. with a standard deviation ±0·37) was obtained from 30 untreated animals. Eight control mice injected with 0·5 ml. olive oil and killed 3–10 days afterwards had an average liver glutathione of 2·68 with a standard deviation ±0·23. In all the 4 mice killed 3–4 hr. after injection with 3:4:5:6-dibenzcarbazole the amount was within the normal range. At the end of 24 hr. the rise was quite definite and the highest values occurred between the 3rd and 8th days. This rise in glutathione, however, did not occur invariably. Among 16 mice killed 48 hr. after injection 4 were within the normal range and 8 were below it. But 31 out of 40 mice killed after 48 hr. showed a glutathione content above the highest limit of the normal range.

The ascorbic acid content of these livers showed no significant change. There was great variation among apparently normal mice (0·152–0·465 mg. per g.) and 59 out of 67 treated animals (86 %) were within this range. In all cases where the glutathione was very low the ascorbic acid was below the normal average. Fat and cholesterol estimations were not carried out in this experiment.

Histological examination of paraffin sections (special methods for staining fats were not used) suggested that many of the livers were either very fatty or contained glycogen, in agreement with the pale colour seen with the naked eye. However, fat or glycogen appeared to have been present in sections of the livers of untreated mice which had been fed on Fox Chow and, as it is impossible to estimate the amount of fat histologically, no conclusion could be drawn. No proliferation of the bile ducts was observed in these mice of which none lived more than 17 days. The most striking feature observed histologically was the differentiation of the liver lobule into an inner zone of well stained cells surrounding the central vein and an outer zone of large degenerate cells which appeared to have contained fat or glycogen. This zoning was observed in less than one-third of the mice and in some of these was very slight. The glutathione content of these has been indicated in Fig. 1, which shows that most of the slightly necrotic livers occurred 24 hr. after the injections and the glutathione in these was above the normal limit. Seven out of 13 livers showing toxic necrosis and degeneration were in mice which had been injected 48 hr. previously, and all of these (except one at 24 hr.) contained less than the normal average of glutathione, and the majority (8 out of 13) less than the lowest normal limit. The most advanced necrosis was seen in two livers at the 4th and 7th days.

None of the livers of mice killed 8 days or more after injection showed necrosis or hepatitis indicating that recovery and regeneration occurred. Many of these livers showed signs of regeneration and growth. With the completion of recovery the glutathione content returned almost to the normal value.

Determinations of the respiration and glycolysis (Table I) show that the four livers with necrotic areas on which these determinations were made had low values for respiration, with Q_{O_2} ranging from $-2{\cdot}3$ to $-7{\cdot}6$ instead of the

Table I. *Livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole*

Time after injection days	Glutathione mg. per g.	Ascorbic acid mg. per g.	Metabolism		Histological appearance
			Respiration Q_{O_2}	Glycolysis $Q_L^{N_2}$	
2	1·22	0·135	− 3·4	+1·4	—
2	3·73	0·310	−14·3	+4·1	—
2	2·44	0·152	− 7·6	+2·0	Intense hepatitis
2	2·02	0·191	− 3·6	+1·6	,,
3	1·23	0·200	− 2·3	+1·3	,,
3	4·47	0·428	−10·8	—	—
3	4·52	0·333	− 9·1	+3·4	—
4	3·70	0·258	− 9·7	—	—
6	4·97	0·285	− 7·0	+3·2	—
6	5·98	0·250	−10·8	+2·3	—
7	0·86	0·106	− 9·2	+6·1	—
7	6·08	0·330	− 5·0	+3·1	—
7	2·82	0·250	− 3·7	+1·3	Intense hepatitis
8	4·82	0·293	− 6·9	+2·5	—
9	4·17	0·292	− 8·6	+1·4	—
10	4·66	0·099	− 7·0	—	—
10	4·62	0·149	− 3·3	+3·9	—
13	1·59	0·038	− 3·2	—	—
13	3·52	0·460	−11·7	—	—
17	4·37	0·234	− 4·8	+3·8	—
17	3·72	0·148	− 2·3	—	—

normal values of -10 to -12. The metabolism of the other livers is on the whole low but irregular.

Series 2. The mortality in the first experiment (Fig. 1) was high; no mice were examined after 17 days, and therefore the chance of any development of

neoplastic changes in the liver was small; hence the dose was reduced to 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse (Fig. 2). Ascorbic acid, glutathione, fat and cholesterol were estimated on all livers in this experiment. Many died (of a series of 65 mice 28 (43 %) were alive 39 days after injection) but many lived for long periods and those which survived for 200 days appeared healthy. One mouse killed on the 176th day after injection had a spindle-celled sarcoma at the site of injection. The livers of many killed after the 40th day showed a nodular appearance and in others the lobes seemed to be swollen and often two or more lobes appeared fused.

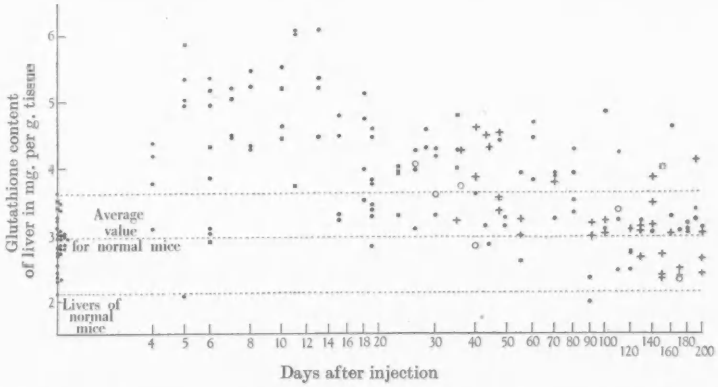
Glutathione content of liver in mg. per g. tissue
6
5
4
3
2
Average value for normal mice
Livers of normal mice
4 5 6 8 10 12 14 16 18 20 30 40 50 60 70 80 90 100 120 140 160 180 200
Days after injection

Fig. 2. The glutathione content of livers of mice injected with 3:4:5:6-dibenzcarbazole (12·5 mg. per kg. body wt.). • Represents liver with normal appearance of bile ducts. o Represents liver with early bile duct proliferation. + Represents liver with advanced bile duct proliferation.

The glutathione contents of the livers are shown in Fig. 2. Again a very distinct increase was observed which was much more consistent than in the earlier experiment. Only three values below the normal average occurred in the 40 days following injection and two of these were close to the normal average. The glutathione content was greatest during the first 20 days and then fell off gradually. Among 34 livers examined between the 120th and 200th days there were only four values above the normal range. The ascorbic acid content lay almost wholly within the wide normal range. The cholesterol and fat contents also remained approximately normal except for a few isolated cases. The cholesterol content varied from 0·216 to 0·645 % except for two livers with 0·169 and 1·075 % cholesterol. The liver fat content ranged from 2·1 to 8·6 % with two exceptions of 1·6 and 11·2 %. The variations in fat and cholesterol could not be correlated with histological appearance. The hepatitis and necrosis which were so striking in the first series were seen in only 7 mice. The instances of early bile duct proliferation and advanced bile duct hypertrophy (the distinction between these two conditions is quite arbitrary) are indicated in Fig. 2. The advanced biliary hypertrophy resembles cholangioma but as transplantation experiments were not performed it is not possible to decide whether this type of growth is malignant or not. No cases of advanced hypertrophy occurred before the 35th day and 33 of 83 mice (40 %) killed after this time showed definite bile duct proliferation. One mouse on the 26th day and another on the

30th day showed indications of the development of this condition. Cholangiomatous growth was observed in livers both with normal and with increased glutathione.

Table II. *Livers of mice injected with 3:4:5:6-dibenzcarbazole (12·5 mg. per kg. body weight)*

Time after injection days	Glutathione content mg. per g.	Ascorbic acid content mg. per g.	Metabolism Q_{O_2}	Metabolism Q_L^N	Histological appearance
10	5·18	0·37	− 8·3	4·9	—
90	1·98	0·51	− 13·5	1·2	—
90	2·33	0·29	− 13·8	3·5	—
163	2·90	0·56	− 8·9	1·5	—
163	3·40	0·42	− 10·4	4·6	—
164	4·05	0·41	− 5·6	2·6	—
164	3·54	0·41	− 8·1	2·1	Advanced bile duct hypertrophy
164	3·54	0·37	− 7·7	4·4	—
172	3·35	0·25	− 14·7	2·0	Advanced bile duct hypertrophy
172	3·55	0·31	− 9·0	2·1	,,
172	3·23	0·36	− 9·6	2·4	,,
176	2·72	0·30	− 7·8	1·1	,,
176	2·15	0·33	− 6·2	4·3	,,
176	4·50	0·17	− 6·8	2·7	,,
176	3·65	0·27	− 7·7	1·3	,,

Table III

Time after injection days	Wt. before injection g.	Wt. after injection g.	Wt. of liver g.	Glutathione mg. per g.	Ascorbic acid mg. per g.	Fat %	Cholesterol %
			Livers of mice injected with 1 mg. methylcholanthrene				
4	23	17·0	0·89	2·16	0·190	3·4	0·37
4	27	19·5	1·12	2·60	0·331	3·8	0·31
4	27	19·5	1·28	2·27	0·336	4·1	0·30
4	23	20·0	1·32	2·60	0·315	4·4	0·27
8	27	21·0	1·18	2·23	0·304	3·6	0·38
8	25	20·5	1·00	2·79	0·325	4·8	0·35
8	18	17·5	1·02	3·29	0·375	3·6	0·28
8	22	17·5	1·10	3·30	0·358	3·5	0·34
12	24	17·5	0·90	2·77	0·272	3·1	0·32
12	21	16·5	0·78	2·40	0·281	3·1	0·40
12	26	22·0	1·05	2·88	0·380	4·1	0·33
12	18	16·5	0·98	3·77	0·370	3·8	0·31
16	20	28·0	2·30	3·37	0·422	2·9	0·31
16	21	27·5	2·16	2·72	0·411	3·0	0·28
16	19	22·0	1·73	3·00	0·400	3·2	0·38
16	16	21·5	1·85	3·80	0·450	4·0	0·32
20	—	26·0	1·66	3·12	0·406	4·0	0·27
20	18	20·0	1·34	2·81	0·433	3·9	0·26
20	—	16·0	0·84	2·50	0·282	3·6	0·32
20	—	20·0	1·46	2·82	0·502	3·8	0·28
			Livers of mice injected with 2 mg. 1:2:5:6-dibenzanthracene				
3	—	—	—	3·00	0·217	2·9	—
4	—	—	—	2·78	0·324	3·4	—
4	—	—	—	3·47	0·291	3·5	—
6	—	—	—	2·50	0·272	3·6	—
6	—	—	—	2·58	0·299	3·0	—
7	—	—	—	1·76	0·328	3·5	—
7	—	—	—	3·63	0·230	5·0	—
10	—	—	—	3·37	0·239	3·5	—

The metabolisms of the livers of some of the mice were determined and the results obtained are given in Table II. The metabolism of livers with advanced biliary hypertrophy is of the same order as that of normal livers.

Series 3. Eight mice which were injected intraperitoneally with 2 mg. 1:2:5:6-dibenzanthracene in olive oil and killed 3–10 days afterwards showed no significant changes in the glutathione, ascorbic acid or fat content of the liver (cf. Table III). No histological examination was carried out.

Twenty mice were injected intraperitoneally with 1 mg. methylcholanthrene dissolved in 0·25 ml. lard per 20 g. mouse and killed at intervals up to 20 days after the injection. The livers of these animals appeared normal except for numerous adhesions. The analytical results obtained on these livers are shown in Table III: no significant changes were observed and histological examination of all the livers revealed no abnormalities.

Discussion

Considerable changes in the livers of mice may occur as the result of a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole (0·25 or 0·5 mg.). Boyland & Brues [1937] found that rats receiving twice weekly subcutaneous injections of 1 mg. of this compound lived for a long time and showed no liver lesions although spindle-celled sarcomata developed at the site of injection, while mice receiving this treatment died after one or two injections. In our own experiments the mortality was also high in mice which were given a single dose of 0·5 mg. 3:4:5:6-dibenzcarbazole and striking histological changes (hepatitis and necrosis) were observed in the livers of many of those killed during 7 days following the injection. It would appear that 3:4:5:6-dibenzcarbazole is more toxic to mice than to rats and this may possibly be associated with its effect on the liver in the mouse. With 0·25 mg. of the compound the mortality was much less and few cases of hepatitis were observed.

The first definite proliferation of bile ducts was observed 35 days after injection of 0·25 mg. although the first indication of such development was seen at the 26th day. These times agree very well with those at which Boyland & Brues observed similar biliary proliferation in mice painted twice weekly with the same compound.

The glutathione content of the liver had increased 24 hr. after injection and was at its maximum during the first 20 days, after which it decreased. Since the ascorbic acid, fat and cholesterol contents remained approximately normal we consider that this is a true increase in glutathione and is not due to a decrease in the water content of the liver. The glutathione content was at its maximum before any neoplastic change was observed (Fig. 2). Some of the "cholangiomata" were seen in livers with an increased glutathione content but most of them occurred when the glutathione had returned to normal.

In the first experiment the livers with highest glutathione content were found after the period when the histological changes were most marked. High glutathione content was often present in those livers in which regeneration was taking place. In about 10 days when the regeneration was complete the glutathione content fell again. In the second experiment, in which the dose of 3:4:5:6-dibenzcarbazole was less, very high values for glutathione content were found during the first 20 days and during this time recovery from the injection with regeneration of cells was probably taking place. Although in many cases the livers were greatly enlarged it was not possible to correlate liver weight with glutathione content. The results indicate that the 3:4:5:6-dibenzcarbazole has

a toxic action which is followed by regeneration accompanied by an increase in glutathione, and at a later stage bile duct hypertrophy takes place.

The decrease in glutathione content which was observed (Fig. 1) in certain mice receiving 0·5 mg. 3:4:5:6-dibenzcarbazole was accompanied by a decrease in ascorbic acid and both the macroscopic appearance of the livers and the study of histological sections suggest that this may have been due in part at least to an accumulation of fat.

1:2:5:6-Dibenzanthracene, which produces hepatoma in mice of the C_3H strain [Andervont, 1937] but has not been shown to produce proliferation of bile ducts, and methylcholanthrene, which is not known to produce either of these changes, did not affect the glutathione, ascorbic acid or fat content of the livers of mice.

Summary

A single intraperitoneal injection of 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse caused an increase in the glutathione content of the liver which was maximal during the first 20 days. This preceded a considerable proliferation of bile ducts which occurred in 40% of the mice examined between the 35th and 200th days. When 0·5 mg. was given there was a similar increase in glutathione content in the majority of the mice but in a few there was a decrease which in most cases was accompanied by a partial necrosis of the liver lobules. This increase in glutathione was not observed in mice injected with 1:2:5:6-dibenzanthracene or with methylcholanthrene. No change in the ascorbic acid or cholesterol of the liver occurred with any of these carcinogenic compounds.

We wish to thank Prof. E. L. Kennaway and Prof. G. R. Cameron for help and advice and Mrs Boyland for determinations of the metabolism of some of the livers. One of us (E. H. M.) is indebted to the Sir Halley Stewart Trust for a fellowship and the research was supported by grants from the British Empire Cancer Campaign. Our thanks are also due to Messrs Roche Products, Ltd., for supplying the 3:4:5:6-dibenzcarbazole.

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CXCIII. THE EFFECT OF VITAMIN E DEFICIENCY ON THE RAT

I. DURATION OF GESTATION

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It has been known for some time [Kirkham, 1916; Brambell, 1937] that if small rodents or shrews are mated during the *post partum* oestrus so that lactation and gestation are simultaneous, then provided that the number of young suckled exceeds a minimum, which is characteristic for each species and is 5 for the rat, gestation is prolonged. There is no direct correlation between the duration of gestation and the number of young suckled, but a rat suckling 9 young may have a gestation period lasting 10 days beyond the normal period.

It has been noticed, in the course of a number of vitamin E assays, that among the proved sterile animals which had been given a small dose of vitamin E, gestation was often prolonged beyond the normal 22 days characteristic of our stock. This prolongation was so consistent among the rats suffering from severe vitamin E deficiency that a further investigation of the problem was undertaken, which has already been the subject of a preliminary communication [Barrie, 1937, 3].

Two diets were used throughout the experiment:

Diet E. 5.	Fat-free casein	2000 g.
	Rice starch	6000 ,,
	Lard	800 ,,
	Cod liver oil	200 ,,
	Salt mixture	500 ,,
	Yeast extract	180 I.U. vitamin B_1 per 100 g. diet

This diet contains no detectable amount of vitamin E and 635 animals fed on it from weaning all resorbed in their first gestation.

The second diet, E. 4, differed from E. 5 in that light white casein was used instead of fat-free casein. However, animals fed on it were usually capable of producing two litters before the resorption typical of vitamin E deficiency occurred. That the E. 4 diet contained inadequate vitamin E, even for the first of these litters, was shown by the fact that the young produced became abnormal and usually died within 23 days after birth [Barrie, 1937, 1, 2].

In the experiments which are reported here, all the test animals were mated with males which had been fed on a diet rich in vitamin E and been proved fertile. Successful implantation of the ova was verified by the finding of the placental sign (detection of blood in the vagina) between the 9th and 13th days after mating. A daily record of the weight and condition of the animals was kept from the occurrence of the placental sign and was continued either until the birth of the young, or until resorption was so advanced that the weight had fallen considerably. In those cases in which young were born, a daily record was kept of the weight and condition of the litter.

The test animals were divided into 5 groups:

1. *Negative controls.* These were not specially selected and were the sterile and untreated representatives of the colony of vitamin E-free rats. These rats were fed on the vitamin E-free diet E. 5 from weaning, and in spite of positive mating followed by successful implantation, the foetuses were resorbed *in utero* in the first gestation.

2. *Animals severely deficient in vitamin E.* This was a group of 22 rats fed on the E. 4 diet until approximately 1 month before mating when they were transferred to diet E. 5. These animals usually produced a living litter but were never able to rear it.

3. *Animals slightly deficient in vitamin E.* This was a group of 40 fed on the diet containing a small but still inadequate amount of vitamin E (diet E. 4). Although able to produce living litters, they were seldom able to rear them.

4. *Positive controls.* This was a group of 27 fed on the vitamin E-free diet (E. 5), but on mating they were given a large dose of a vitamin E concentrate. The adequacy of the dose was shown by the ability of the doe to rear her litter.

5. *Stock rats.* This was a group of 50 rats selected at random from our normal breeding stock. They were fed on a diet containing an adequate amount of vitamin E. These animals all reared their litters and the few deaths which occurred among the young were usually due to the mother becoming fierce and killing them.

Table I

Group	No. rats in group	Length of gestation (days) Average	Longest	Shortest	No. young born	No. born dead	No. young reared
2	22	25·3 (corrected 24·8)	32	23	71	43	1
3	40	23·0 (corrected 22·5)	25	19	242	24	62
4	27	23·04 (corrected 22·54)	25	22	175	17	119

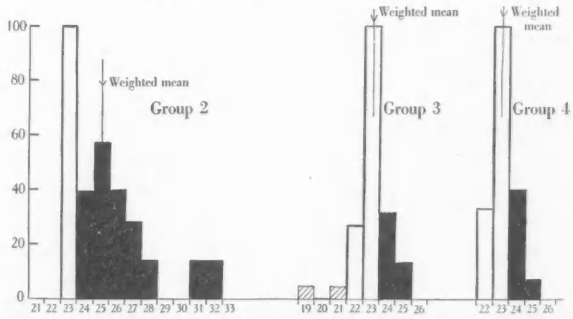

Fig. 1. Plotted to show the relative distribution of the gestation period. The largest group was arbitrarily selected as 100 in each case. The hatched columns represent gestations which were shorter than normal, the hollow columns those of normal duration, and the black columns those in which the duration was beyond the normal range.

Table I gives the length of gestation and the live and dead litter rates in animals of groups 2, 3 and 4. Since the animals were weighed and examined only once daily, at approximately 11 a.m. on each day, the litters born were anything up to 24 hr. old when first observed. In order to correct for this, one half day has been deducted from the average gestation period for each group. It will be seen that the positive controls had a gestation period varying between 22 and 25 days with an average of 23 days, or 22·5 after making the correction. This

period is the same as that of our normal breeding stock (see also Hain [1934]). The normal range must, however, be taken to lie between 21 and 23 days, as a variation of one day could not be considered abnormal.

The animals which were suffering from a slight deficiency of the vitamin (group 3) had a gestation period of 19–25 days, 67·5 % of them falling within the normal range of 22–23 days; those of group 2, which were suffering from a much more severe deficiency of vitamin E, had a gestation period which lasted as long as 32 days in one case, and the duration was normal in only 31·82 % of the animals. Fig. 1 shows these results scaled down so that the relative spreads in each group can be compared. The weighted mean of groups 3 and 4 is 23 days, while that of group 2 is 25·3 days (both uncorrected).

These results give a definite indication that the length of gestation bears an inverse relation to the amount of vitamin E in the animal's diet.

Although the majority of the animals of group 3 which were suffering from a slight deficiency of vitamin E had a gestation period of normal duration, their deficiency of vitamin E was shown by the low survival rates of the litters.

Table II

Mother	No. of litters averaged	Av. no. born per litter			Av. no. reared
		Total	Born alive	Born dead	
Group 5	50 First litter in each case	7·84	7·84	0·00	7·24
Group 4	27	6·48	5·87	0·63	4·40
Group 3	40	6·05	5·99	0·06	1·55
Group 2	22	3·23	1·28	1·95	0·04
Group 1	635 pregnancies	0·00	0·00	0·00	0·00

Table II gives the average number of young born to animals in each of these groups, and again a difference is seen: the animals receiving adequate vitamin E (group 4) had litters averaging 6·5 young with an average of 0·6 still-births per litter. Those receiving less vitamin E (group 3) had litters of approximately the same number, but the still-birth rate was slightly higher and the litters were not reared, while the animals of the second group which had not received any vitamin E for a month before mating had very small litters averaging 2·14 live- and 1·27 still-births per litter. In spite of the smaller number born, the mothers were unable to rear them. Among our normal breeding stock, which is fed on a diet rich in vitamin E, we find that the average number of young born is 7·84 and in the 50 selected at random there were no still-births. Among these normal breeding stock rats there are a few which never give birth to more than 5, even in the first gestation, while others have as many as 14. Old breeding rats frequently have small litters, but if the number born is fewer than 5, we consider the animal unsuitable for breeding. Hence it may be concluded that if a vitamin E-deficient rat is dosed with vitamin E and has a litter of 5, the dose given is not necessarily smaller than that which produced 7 in another rat, but if the number in the litter is less than 5 and the animal is young and in good condition, there is an indication that the dose given was inadequate; this supposition is borne out by the fact that such small litters are seldom reared by vitamin E-deficient rats.

Fig. 2 shows typical weight curves (*A*) of a resorbing rat, (*B*) of a rat given a large dose of vitamin E and (*C*) of a rat suffering from severe vitamin E deficiency. These three rats were chosen because they were all at approximately the same weight on the 13th day of pregnancy. The three curves are very similar

until the 16th day, from which date the dosed rat (136) gained weight until full term. This weight curve is typical of those given by the 27 positive controls. The resorbing rat and the rat partially deficient in vitamin E ran together until the 17th day; the latter rat then started to gain weight more rapidly until the birth of the litter on the 32nd day of pregnancy.

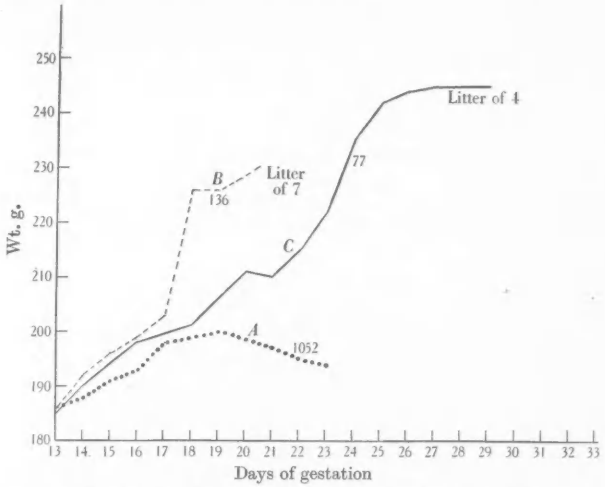

Fig. 2. Rat 136 dosed with vitamin E; rat 77 given inadequate vitamin E; rat 1052 given no vitamin E.

A pregnant rat deficient in vitamin E, which is also receiving a diet containing no vitamin E, gains weight slowly from the time of implantation until about the 15th day; after this stage the weight gradually decreases, and there may be a haemorrhage, possibly comparable with abortion, at any time from the 16th day onwards (Fig. 2, rat 1052). Only one rat has been taken as an example, as the rats become pregnant at different weights and it would not be desirable to make a composite curve. The curve for this single rat agrees with that of Evans [1927] and is typical of over 800 obtained during this and other experiments. Placentation is often early in these rats and if it is detected on the 9th day of gestation and is followed by continuous bleeding it is fairly certain that resorption will follow; in fact, the time and type of placental sign found give a fairly accurate forecast of the course of pregnancy. Tables III, IV and V illustrate this point.

Rat 697 is representative of group 4, rat 658 had very little reserve of vitamin E and is therefore comparable with rats of group 2, while rat 115 had been fed on the vitamin E-free diet from weaning and belonged to the negative control group 1. Comparison of these three records shows that when pregnancy takes a normal course there is very little bleeding.

To the rat whose gestation period lasted 32 days, only 4 young were born and these were well above the average birth weight, weighing 8 g. each instead of the normal 4·5 g. In a few cases, animals have died or have been killed at some stage during the pregnancy and the *post mortem* findings have given the explanation of this prolonged gestation. One of these animals was 19 days

Table III. *Rat 697 fed on vitamin E-free diet, but given a dose of 2·5 ml. vitamin E concentrate on mating. This concentrate was known to be active in a dose of 1·5 ml.*

Stage of pregnancy days	Wt. g.	Vaginal smear
13	184	Faint trace of blood
14	184	Mucus and blood
15	186	Mucus and blood
16	189	No blood
17	191	No blood
18	196	No blood
19	202	No blood
20	211	Trace of blood. Foetuses could be felt
21	220	More blood than preceding day
22	222	Unchanged
23	229	Occult blood, no fresh blood
24	Litter of 6	—

Table IV. *Rat 658 fed on vitamin E-rich diet for 4 months then on vitamin E-free diet for 3 months before mating. The animal had practically run out of vitamin E*

Stage of pregnancy days	Wt. g.	Vaginal smear
10	188	Trace of blood
11	189	Trace of blood
12	Not examined	—
13	187	Trace of blood
14	195	Mucus and blood
15	196	Very faint trace of blood
16	200	Fresh blood
17	202	Very faint trace of blood
18	206	Quite a lot of blood
19	Not examined	—
20	203	Mucus
21	212	Mucus
22	216	Trace of blood. Foetuses felt
23	221	Faint trace of blood
24	224	Faint trace of blood
25	Litter of 3 (2 dead)	—

Table V. *Rat 115 fed on vitamin E-free diet from weaning. First pregnancy*

Stage of pregnancy days	Wt. g.	Vaginal smear
10	180	Trace of blood
11	180	Trace of blood
12	Not examined	—
13	183	Trace of blood
14	180	Large amount of blood
15	186	Large amount of blood
16	181	Fresh blood
17	178	Bleeding profusely
18	176	Blood and mucus
19	Not examined	—
20	175	Bleeding profusely
21	175	No blood
22	176	Fresh blood
	Litter resorbed	

pregnant when it died and the remains of 7 resorbing foetuses could be seen together with two apparently normal but very undersized ones which would probably have been born at some time after 21 days' gestation. Similar conditions have been seen in animals which have reached the 24th day of gestation. In a preparation made from a 23 days' pregnant vitamin E-deficient rat which had been given a small dose of vitamin E, resorption of three foetuses was well advanced in the right horn of the uterus, while in the left horn two others were developing, and one was probably being resorbed. This evidence, together with that of the weight curves (Fig. 2), shows that resorption of the litter is not an "all or none" process and that there are varying degrees of vitamin E deficiency. Partially deficient animals resorb some of their foetuses and develop the others so that the number carried to term is smaller than would normally be the case.

It seems probable that owing to the process of resorption, the development of the remaining foetuses is retarded and in consequence pregnancy is prolonged. This is shown very clearly in Fig. 2 where the weight curve of the rat which had a 32 days' gestation first follows the line of the resorbing rat and then leaves it to make normal daily increases in weight until the birth of the litter.

Similar prolongations of gestation have been observed before in the rat, the mouse and the shrew, but under very different circumstances. Kirkham [1916; 1918] found that this prolonged gestation in the mouse was associated with delayed implantation and that the retardation occurred in the blastula stage of the embryo. There was no evidence of resorption of any of the foetuses. The phenomenon is therefore hardly comparable with that observed in the vitamin E-deficient rat, in which implantation is often earlier than normal and is followed by resorption of some of the foetuses. The prolonged gestation in both these and the lactating animals is very probably due to some impairment of the normal function of the corpus luteum. It is here that a connexion between the two sets of results seems most probable, if the function of the anterior pituitary gland is controlled by vitamin E. This theory is strongly supported by the work of Rowlands & Singer [1936], who showed that the luteinizing activity of the pituitary of vitamin E-deficient rats is about half that of the normal rat. Verzár & Kokas [1931] showed that the coat of the vitamin E-deficient male rat changed to the type characteristic of hypophysectomized animals and later Verzár [1937] summarized the published work on vitamin E which gives support to this theory. Nelson [1933] found castration changes in the anterior pituitary of vitamin E-deficient male rats and Barrie [1937, 2] described degranulation of the acidophils and basophils of the anterior pituitary of the vitamin E-deficient female rat and ascribed the abnormal condition of the young born to partially vitamin E-deficient rats to anterior pituitary involvement. It can therefore be taken that the vitamin E-deficient animals used in these experiments, in which gestation was prolonged, were suffering from anterior pituitary deficiency. It seems probable that this deficiency was sufficiently severe to impair the function of the corpus luteum, although so far no morphological evidence of such impairment in vitamin E deficiency has been found.

Summary

In the rat partially deficient in vitamin E the length of gestation bears an inverse relation to the amount of vitamin E in the diet. Such partially deficient animals are unable to develop all the ova implanted, with the result that some of the foetuses are resorbed *in utero*.

It is suggested that the prolongation of gestation caused by vitamin E deficiency is due to the resorption of some of the foetuses delaying the development of the others and that this condition is an indirect effect, through the ovary, of anterior pituitary deficiency.

I wish to acknowledge the help of my assistant, Miss I. Harley, throughout the experiments. My thanks are also due to Dr F. H. Carr and Dr S. W. F. Underhill for their encouragement, and to the directors of The British Drug Houses, Ltd., for permission to publish this work.

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CXCIV. THE EFFECT OF VITAMIN E DEFICIENCY ON THE RAT

II. LACTATION

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(*Received 17 June 1938*)

The accepted characteristic of vitamin E deficiency is resorption of the foetuses *in utero* before the completion of gestation. In ordinary circumstances, absolute deficiency of a vitamin must be a very rare occurrence and for this reason the characteristics of partial deficiency are probably more readily observed.

Vitamin E is in some way connected with the normal function of the anterior pituitary gland [Verzár, 1937; Barrie 1937, 1] and when one considers the diversity of function of this gland it is not surprising that the effects of even a partial deficiency of the vitamin are widespread.

If a female rat is fed on a diet which contains inadequate vitamin E, she may produce a living litter after mating with a normal male but is unable to rear it because the young become abnormal and die within 21 days of birth. This abnormality was described in an earlier paper [Barrie, 1937, 1] and an apparently similar condition was observed by Evans [1928] and found by him to be preventable if the young were dosed with wheat germ oil within 15 days of birth. In a preliminary communication [Barrie, 1937, 2] the result of an experiment was given in which it was found that this abnormality of the young, the onset of which occurred about 18 days after birth, was due to some deficiency in the mother's milk. The experiment has been repeated and confirmed.

Rats which had been proved to be sterile were mated and given a small dose of a vitamin E concentrate on the day of mating. The activity of the concentrate used had been previously determined and the dose given was known to be adequate for the production of a litter, but inadequate for the rearing of it. In one experiment the litter was exchanged for a normal stock litter born on the same day, in another experiment the exchange was made at 3 days, and in further experiments at 5 and 6 days after birth. In every case it was found that the animals fed by the vitamin E-deficient mother became abnormal, while those fed by the stock mother grew normally.

Full details of the first of these interchange experiments are given in Table I.

On the 16th day the young fed by the vitamin E-deficient rat were all definitely affected, although her own males were weaker than the females she was fostering. At this stage of the experiment the stock mother's own males were killed as she was a very small rat and a litter of 10 seemed too much for her; she was therefore left with only the fostered females from the vitamin E-deficient rat. At 17 days the young fed by the vitamin E-deficient rat were definitely abnormal; they were dragging their hind legs and their forepaws were contracted and on the following day the condition of the males was so bad that they were killed. The fostered females reached the same condition on the 21st day but the females born to the vitamin E-deficient mother and fed by the stock mother grew normally and were weaned when 21 days old.

Table I. *Rat 214, partially deficient in vitamin E; litter of 8, 3 ♂ 5 ♀; total wt. 43 g. 6 days after birth total wt. 77 g., all apparently normal. The 5 ♀ exchanged for 4 stock ♀ born on the same day; the vitamin E-deficient mother then had 4 stock ♀ and her own 3 ♂, the stock mother had 5 vitamin E-deficient ♀ and her own 5 ♂*

Days after birth	Fed by vitamin E-deficient mother		Fed by stock mother	
	Wt. ♂ g.	(Fostered) Wt. ♀ g.	♂	(Fostered) Wt. ♀ g.
6	10 10 10	11 11 11 11	Not weighed	10 9 9 10 9
9	14 13 14	12 12 12 13		12 12 12 12 12
14	19 19 19	18 18 18 18	Smaller but more lively	17 18 18 17 18
16	20 20 20 One has weak hind legs Another has front paws contracted	20 19 20 20 Two have weak hind legs	♂'s killed because the litter was too large	17 18 19 16 18 Normal Normal
17	22 22 20 Two very abnormal*	22 22 22 22 No change		
18	19 22 18 Condition much worse, killed	23 23 23 23 All have weak hind legs		18 20 20 18 18 20 All active and normal
21		32 27 25 1 dead others very weak and dragging hind legs		25 26 27 24 27
22		All dying, killed		28 28 31 26 29 Normal, weaned

* Fur soft and cottony, hind legs dragging, fore feet contracted; animals very weak.

A very similar result was obtained when the exchange was made at 5 days, but in this case the males and not the females were exchanged. The result was further confirmed by giving to another vitamin E-deficient mother 4 stock young in addition to her own 2. The young were 3 days old when given to the vitamin E-deficient mother and they and her own litter became abnormal and died when between 20 and 22 days old.

In a further case a vitamin E-deficient rat had a litter of 6 which died when 12 days old and the mother was given 2 stock rats, aged 8 days, to foster. Both of these were reared.

In a somewhat similar experiment a sterile vitamin E-free rat was dosed with vitamin E concentrate though it was still fed on the vitamin E-free diet. This rat had a litter of 8, 5 of which were born dead, and one of which died later, leaving 2 which grew very slowly and died when 16 days old. The mother was given 4 stock young aged 3 days which she fed for 8 days, then 2 of them were returned to their own stock mother and 3 other stock young, aged 4 days, were given to the vitamin E-deficient mother, who was thus fostering 2 stock rats aged 11 days and 3 aged 4 days. The older two developed normally, as did the two which had been returned to their stock mother after being fostered by the vitamin E-deficient mother for 8 days. The 3 younger animals fostered by the vitamin E-deficient rat developed the characteristic leg weakness when 18 days old.

The exchange experiments show that the milk of the vitamin E-deficient rat is lacking in some substance essential for the normal growth and development of the suckling young, but that if the young are fed on normal rat milk until they

are 8 days old they obtain sufficient of this substance to last them through the rest of their suckling period. In view of the experiments of Evans [1928], in which he found that the onset of muscular weakness in the young was prevented if they were dosed with a source of vitamin E before they were 15 days old, it can safely be presumed that this missing factor in the milk is the vitamin, and the foregoing experiments suggest that the young of these deficient rats are normal at birth since they can be reared by a normal mother receiving vitamin E in her diet. The subsequent history of these and other fostered rats is being followed and will be the subject of a future publication.

In all the experiments the mother was fed on a vitamin E-free diet throughout but was dosed with just sufficient vitamin E on mating to enable her to produce a living litter. During the gestation and lactation periods the animal received no further dose of the vitamin. In one experiment the animal had been given a larger dose of vitamin E on mating, yet was unable to rear her own litter, 5 of which were born dead, one of which died soon after and the remaining 3 of which were weaklings and died when 16 days old. At this stage the mother evidently had sufficient vitamin E in her milk to fulfil the requirements of normal healthy sucklings for she was able to rear two stock young which she fostered, but this evidently exhausted her store of vitamin E as she failed to rear a further 3 given 8 days later.

From this it may be inferred that vitamin E is not stored for any length of time. In this case the dose given was large but the animal had exhausted her supply by the time she was feeding the third litter. If, however, the dose given is large enough, the animal stores sufficient vitamin E to enable her to complete her pregnancy and to feed and rear her litter. This was shown by an experiment in which two normal stock rats, which had been fed from weaning on an adequate diet containing plenty of vitamin E, were mated and from the day of mating fed on the vitamin E-free diet; both rats had normal gestations, one had a litter of 10 and the other 11. Both fed and reared their litters so that they must have stored sufficient vitamin to carry them through this period. The experiments previously described showed that at birth a rat, whether born of a normal or of a vitamin E-deficient mother, has no store of vitamin E, or so little that it is insufficient to carry it through the suckling period. If this is the case then the abnormality which becomes obvious at about 18 days after birth must be due to vitamin E deficiency and should, if taken in the early stages, be curable by this vitamin. To test this point a sterile rat was mated and given a dose of vitamin E concentrate and had a litter of 6, one of which died. Four of the remaining 5 were given small doses of an active oil from the 6th day onwards. On the 16th day the negative control was weak and the hind feet were contracted while the dosed animals were normal. The negative control became progressively worse; its front feet were affected on the 17th day and on the 21st day it was in a very weak condition, was unable to move and had lost weight. At this stage it was given 0·06 ml. of an oil active in the adult rat in a dose of 0·8 ml.; the following day its condition was worse. The dose was repeated on the 23rd day and on the 24th day there was a marked improvement. On the 25th day the animal was moving normally. No further dose was given and the rat was put on the vitamin E-free diet and died 4 days later. The animals which were dosed from the 6th day onwards remained normal throughout. The experiment was repeated with another litter, but in this case the negative control was dosed 2 days after the onset of the leg weakness and it made a complete recovery.

In another experiment a litter of 9 was born to a vitamin E-deficient mother and was left untreated until 3 days after the onset of the symptoms. At this stage

6 of the litter were alive and all were badly affected, showing both fore and hind leg weakness. All 6 were given several doses of 0·06 ml. of a vitamin E concentrate of which the adult rat dose was 0·31 ml. The details of the experiment are given in Table II.

Table II. *Progress of abnormal young rats dosed with vitamin E concentrate*

Days after birth	No. of animals	Wt. (g.)	Dose (ml.)	Condition
21	6	28–32	0·04	All badly affected, legs weak, feet contracted, hind legs dragging
22	6	—	0·04	Unchanged
23	6	—	0·04	,,
24	6	—	0·06	5 showed improvement, 1 recovered
26	6	26–36	0·06	1 still very bad, 2 practically recovered, 1 improved but feet still contracted, 2 normal
27	6	26–43	0·06	2 unchanged, 1 still weak in left hind foot, 3 normal
28	4	28–45	—	Normal, put on vitamin E-free diet
	2	—	0·06	Still slightly weak
29	2	31 and 50	0·06	Unchanged
30	2	33 and 52	0·06	Slight improvement
31	2	—	0·06	,,
33	2	46 and 60	—	Normal, though left hind foot of one still contracted
34	2	—	—	Both put on vitamin E-free diet, the affected animal gradually regained use of left hind foot

This result was repeated and confirmed with a litter of 6 and again with the 5 survivors of a litter of 7 of which 4 recovered within 10 days and the 5th died on the day following the first dose.

Discussion

Both Evans [1928] and Morelle [1931] described a paralysis in the suckling young of rats deprived of vitamin E. They found that the paralysis affected the hind but never the fore limbs. The mortality was approximately 30%. Evans found that the condition was preventable if the young were treated with a vitamin E concentrate before they were 15 days old. Morelle found that it was prevented by the addition of vitamin E to the mother's diet up to 10 days after mating. Evans made no histological examination, but Morelle examined the nervous system and could find no degeneration. The animals described by Barrie [1937, 1] were obviously comparable, although the condition was much more severe and affected both fore and hind legs and caused carpopedal spasm and convulsions in the final stages. The mortality among these animals was 100%. As in the animals described by Morelle, no nervous degeneration was found, but there was thyroid hypoplasia and involvement of the anterior pituitary.

The present series of experiments, together with the work of Evans and Morelle, shows quite clearly that this paralysis is due to deficiency of vitamin E. The young are apparently normal at birth and under normal conditions would obtain the amount of vitamin E they require from the mother's milk, but when the mother is deprived of the vitamin she does not secrete it in the milk, and unless the young are dosed with vitamin E they suffer from its deficiency and paralysis ensues. This paralysis cannot be caused by any severe lesion as it is fairly readily cured by administration of a large dose of the vitamin. It is

reasonable to suppose that the mode of action of vitamin E is the same in the young as in the adult rat, in which case this paralysis is very probably connected in some way with failure of the function of the anterior pituitary.

It has been known for some time [Evans, 1924; Sure, 1926; Barrie, 1937, 2] that deprivation of vitamin E may cause total or partial failure of lactation, but this failure cannot be due to total lack of milk since the young in the present experiments have all been fat and those that have died have been examined and their stomachs have been found to be full of milk. It may therefore be concluded that although vitamin E in the mother's diet may influence the amount of milk secreted, even if the total amount of milk is adequate still more of the vitamin must be added to the diet before the amount secreted in the milk will be adequate for the requirements of the young.

A very interesting experiment in this connexion was performed by Müller [1936], who found that if sterile vitamin E-deficient rats were fed on human milk the sterility could be cured if the mothers from whom the milk was obtained had been fed on a diet containing vitamin E. This shows that the secretion of vitamin E in human milk is governed by the same factors as in the rat, and although a large volume of clinical evidence for the efficacy of vitamin E in the treatment of habitual abortion is now accumulating, it may well be that this vitamin has a far greater range of clinical application.

I wish to acknowledge the help of my assistant Miss I. Harley, throughout the experiment. My thanks are also due to Dr F. H. Carr and Dr S. W. F. Underhill for their encouragement, and to the directors of the British Drug Houses Ltd., for permission to publish this work.

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CXCV. THE VITAMIN B_2 COMPLEX

VIII. FURTHER NOTES ON "MONKEY PELLAGRA" AND ITS CURE WITH NICOTINIC ACID

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(*Received 23 July 1938*)

At present three diseases in experimental animals are known which are analogous with human pellagra. These are canine blacktongue [Goldberger *et al.* 1928], "monkey pellagra" (described in Part VII of this series [Harris, 1937, 1]) and pellagra in pigs [Birch *et al.* 1937]. The discovery by Elvehjem *et al.* [1937; 1938] that nicotinic acid (or amide) cured blacktongue made it desirable that confirmatory experiments should be carried out on the other species. Since tests on "monkey pellagra" were being continued in this laboratory at the time when the preliminary note of Elvehjem and his collaborators appeared, the effect of nicotinic acid was examined without delay. As is now well known, nicotinic acid has in fact been found effective in curing pellagra in pigs [Chick *et al.* 1938], and in human beings [e.g. Fouts *et al.* 1937; Smith *et al.* 1937; Spies *et al.* 1938]. So far as monkeys are concerned only a brief preliminary note about the observations with nicotinic acid has as yet appeared [Harris, 1937, 2]; the fuller experimental details are therefore set out in the present communication.

Experimental

Animals used. The monkeys used (see Fig. 1) varied considerably in size and age, several of them being young animals still rapidly growing, others being nearly full size. Two among the larger ones had been in continuous use since October 1935, first in the experiments reported in the earlier paper and then in later work undertaken in the interim. One of these two, no. 3, had been formerly a positive control receiving yeast (Fig. 1 in Part VII); the other, no. 6, had received in turn numerous curative supplements each followed by a period on the deficient diet (Fig. 4 in Part VII).

Basal diet. This was the same as that described in Part VII and consisted of a modification of Goldberger's blacktongue diet, supplemented with "radiostoleum" (for vitamins A and D) and orange juice (for vitamin C and possibly other factors needed by monkeys). This basal diet has more recently been given baked in the form of a cake. In the present work extra vitamin B_1 (10 I.U. daily per kg. body weight) and lactoflavin (60γ daily) were also provided, as an additional safeguard against the possibility of partial deficiencies of these factors.

Doses of nicotinic acid tested. Various graded allowances of nicotinic acid were tested at intervals, including 30 mg., 15 mg., 10 mg., 5 mg. and 2 mg. per day. The corresponding responses are best judged from the weight curves (Fig. 1).

Symptoms of deficiency. As was shown in the previous paper, monkeys kept on such a basal diet throve provided that a supplement known to contain the P.-P. factor were allowed (e.g. Eli Lilly "343" liver extract powder, or yeast), or if whole wheat were substituted for the maize. Without such supplements the

animals fell ill with the characteristic symptoms of "monkey pellagra"—including loss of appetite, diarrhoea, vomiting, emaciation—and lost weight rapidly and died. No severe skin lesions were noted, but there was often considerable denuding of the fur. The same symptoms were seen again in the present work in those animals receiving no nicotinic acid, or during those periods when it had been withheld for a time. We have also in the later work been specially struck by the anaemic appearance of the animals, the skin becoming very pale in contrast to its normal pink to bluish appearance. Another symptom was the obvious nervousness or "jumpiness" of the deficient animals. Any detailed account of the pathology of the avitaminosis as seen in monkeys is, however, best deferred to a later paper, and for present purposes we may be content to judge the effectiveness of the nicotinic acid by the weight charts. The recovery of good spirits and of normal alertness, as well as the improvement in appearance and loss of symptoms brought about whenever a source of the anti-pellagra factor was given, were always found to be marked by a corresponding gain in the weight curves.

Results with nicotinic acid. Without exception, the administration of nicotinic acid, in doses of between 10 and 30 mg. per day, was followed by a dramatic cure. Even animals which seemed severely ill as a result of the deficiency quickly responded. Appetites were restored and growth was resumed with no apparent lag.

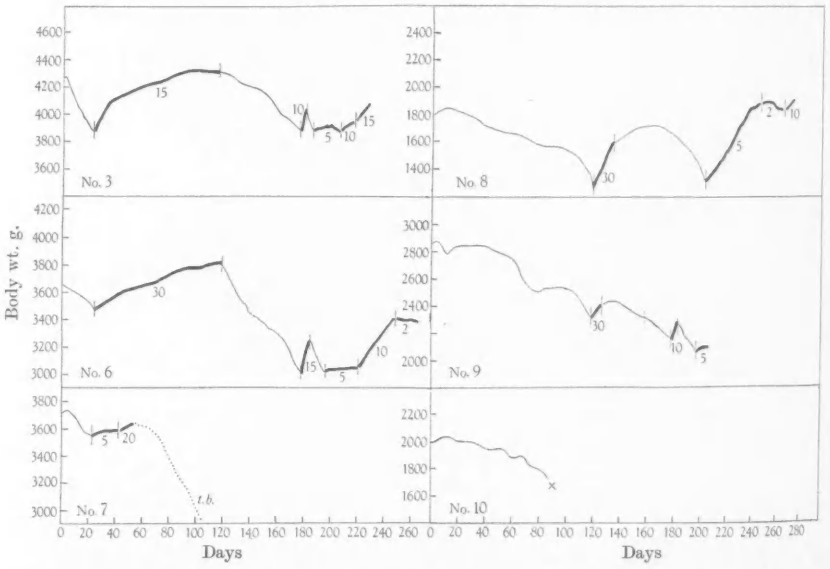

Fig. 1. Growth curves of monkeys. —— Pellagra-producing diet alone. —— Supplemented with nicotinic acid. The numbers under the curve refer to the dose of nicotinic acid given in mg. per day.

(The dotted line shows the rapid loss in weight of a monkey which on autopsy was found to have been suffering from tuberculosis.)

The effect on the weight curve and on the liveliness of the monkey was generally already clearly marked on the next occasion when it was weighed, sometimes as soon as 2–3 days after the start of the dosing.

Minimum curative dose. Judging from Fig. 1 a daily dose of about 2 mg. of nicotinic acid is not quite sufficient for rapid recovery in an animal weighing about 2 kg., but doses of 5 mg. and upward are adequate (animal no. 8, cf. also no. 9). For larger monkeys weighing about 4 kg., doses of 5 mg. seem barely enough, but doses of 10 mg., 15 mg. or 30 mg. suffice (monkeys nos. 3, 6 and 7).

These doses may be compared with those required by other species. Young pigs weighing on the average 30–50 lb. have been protected with doses of 25–60 mg. per day [Chick *et al.* 1938], and human beings have been cured with doses of between 60 and 1000 mg. per day [references as on p. 1479]. The minimum requirement for a dog is thought to be about 0·5–1·5 mg. per kg. per day [Elvehjem *et al.* 1938].

Experiments with nicotinamide and β-aminopyridine [cf. Subbarow *et al.* 1938] are still in progress.

Summary

In tests which have been continued for a period of over eight months nicotinic acid has been found consistently effective in curing "monkey pellagra". The curative dose is of the order of 5 mg. for a monkey weighing about 2 kg., or 10 mg. for a monkey weighing 4 kg.

I am greatly indebted to Mr S. A. Cresswell for his careful management of the monkeys.

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CXCVI. CHEMICAL NATURE OF THE ULTRA-MICROSCOPIC PARTICLES OF SERUM

By ROBERT NEIL CUNNINGHAM and BENJAMIN ALFRED PETERS

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(*Received 23 July 1938*)

McDonagh [1927] first investigated chemically the fine particles in active Brownian movement which are visible in serum under dark-ground illumination; he termed them "protein particles", and drew attention to their behaviour in an electric current and the modifications in their number during various diseases and recovery therefrom. Peters [1936] confirmed these observations, but did not agree with McDonagh's conception that they were protein, on the ground that normal serum proteins, when fully hydrated, should have a refractive index so near to that of water that the particles would not be visible under the ultra-microscope. He further showed that dehydration and precipitation of the serum proteins with alcohol caused an enormous increase in the number of particles visible. Some lecithin and cholesterol determinations in connexion with this work showed little correlation with the number of particles, yet nevertheless on the evidence then available he favoured the hypothesis that the particles were lipin rather than protein.

More recently, Frazer & Stewart [1937] have published the results of a series of investigations on the effects of feeding on the particle content of the serum of human subjects. They found that the particle content of the serum exhibited a considerable increase following ingestion of fat, but was apparently unaffected by protein or carbohydrate meals, and on this physiological evidence concluded that the particles were lipin in nature, although probably enclosed in an adsorbed protein film.

The present experiments were undertaken in order to investigate the nature of the particles by purely chemical and physico-chemical means, which appeared to be a more direct method of approach than physiological methods, and with a view to finding a chemical explanation of the variations in disease noted by McDonagh and Peters as indicated above.

Experimental

The serum employed was pig serum, which is comparable with human serum as regards particle content, and which could be obtained easily from the slaughter-house on the private farm of this hospital. The serum after separation was preserved with 0·5 % NaF, which was found not to affect the particle content.

The microscope was a dark-ground condensing instrument of the ordinary pattern, but the stage was not fitted with a counting-square. Consequently it was not easy to take a direct count of the numerous and rapidly moving particles, and estimations of the particle content were based on observation of the average apparent distance (in mm.) between neighbouring particles as seen through the eyepiece and compared with the scale on the travelling stage.

Frazer & Stewart [1937] had noticed two distinct types of particles, appearing brightly and dully illuminated respectively. In our experiments we have confirmed and extended this observation, distinguishing three types, viz.: large bright ("LB"), medium dull ("M"), and scarcely visible small dull ("S") particles, which are indicated as such in our results; where more than one type is observed the more numerous of the two appears first, whilst the presence of all three with none predominating is indicated by "(mixed)".

In our tables, apparent distances between particles of 1 mm. or less are indicated by "+++" (occasionally "++++" is used to show a difference between two results of this order), 1–3 mm. by "++", 3–5 mm. by "+", and distances greater than 5 mm. by "±". The "LB" particles, which often showed variations not common to the three types, were on such occasions estimated separately; the above system applies there with the distances increased roughly five times.

Fresh untreated serum generally showed a high content of particles of all sizes. The particle content was unaltered by dilution with 0·9% NaCl up to three times the original volume, or by prolonged centrifuging in a laboratory (Martin) centrifuge. Table I shows typical examples of sera, and also the effect of dilution. Two slides were usually made of each specimen, sometimes even more.

Table I. *Particles in normal serum. Effect of dilution*

Selection of typical sera from different experiments.

Serum no.	Content	Size of particles	LB
1	++	M	
2	++	S—M	
3	+++	(Mixed)	+
4	++	(Mixed)	+++
5 (undiluted)	++	(Mixed)	
5 (diluted with ½ vol. 0·9% NaCl)	++	(Mixed)	
5 (diluted with 1 vol. 0·9% NaCl)	++	(Mixed)	
5 (diluted with 2 vol. 0·9% NaCl)	++	(Mixed)	

Precipitation with ammonium sulphate

Serum was half-saturated with ammonium sulphate and the mixture centrifuged. The globulin content of the serum was low, and very little or no precipitate was produced. The amount of ammonium sulphate was later increased to 60 and 70% saturation. Table II shows a typical result.

Table II. *Serum particles and precipitation with ammonium sulphate*

	Before centrifuging		After centrifuging	
% Saturation	Content	Size of particles	Content	Size of particles
(Original serum)	++	M	++	M
50	++	M	++	LB—M
60	+	LB—M	+	LB—M
70	++	M	+	M

Some of the uncentrifuged mixtures showed aggregates of globulin.

It is evident from these results that incomplete saturation of serum with ammonium sulphate does not remove the particles from suspension. Adjustment of the serum to *p*H 4·2 (at which point, as will be shown later, the number of particles shows an apparent maximum) had no effect on their precipitability. Prolonged centrifuging was also ineffective.

The small precipitates produced by incomplete saturation with ammonium sulphate were examined suspended in a small volume of 0·9% NaCl. A few particles were generally observed, but settled rapidly on the slide. Organic solvents failed to dissolve any of the solid material.

Complete saturation of the serum with ammonium sulphate precipitated the proteins entirely; the supernatant liquid obtained by centrifuging these off proved to be optically empty, showing that the particles were removed together with the albumins. Portions of the dried precipitate, extracted with ether or chloroform, failed to yield a detectable quantity of cholesterol.

Sodium chloride, substituted for ammonium sulphate in a few experiments, produced results similar to the above.

Effect of p*H on the particle content*

Portions of 10 ml. of serum from the same source were adjusted to *p*H values over the whole range from 3·0 to 10·0 with the aid of a capillator. The range 3·0–5·0 was examined in detail, since serum proteins are precipitated by acids. Table III shows typical results. The salt concentration was maintained constant by the use of a *N* solution of HCl in 0·9% NaCl. The serum mixtures were examined before and after centrifuging.

Table III. *Variation of the particle content with* p*H*

*p*H (Original serum)	Content before centrifuging	Content after centrifuging	*p*H (Original serum)	Content before centrifuging	Content after centrifuging
	+ +	+ +	5·0	+ +	+ +
3·0	+ +	+ +	6·0	+ +	+ +
3·2	+ +	+ +	7·0	+ +	+ +
3·4	+ + +	+ +	7·6	+ +	+
3·6	+ + +	+ +	8·0	+ +	+
3·8	+ +	+ + +	9·0	+	+
4·0	+ +	+ + +	9·2	+	+
4·2	+ +	+ + +	9·4	±	±
4·4	+ + +	+ + +	9·6	±	±
4·6	+ +	+ +	10·0	±	−
4·8	+ +	+ +	11·0	−	−

Particle sizes at all *p*H values (Mixed).

It is seen from these results that the particle content shows a small maximum at *p*H 4·2–4·4, and on the alkaline side it falls steadily to negligible values at *p*H 10·0–11·0. Centrifuging at any *p*H removes very few of the particles if any.

Serum was next incubated at the point of maximum precipitation (*p*H 4·2–4·4) for 12 hr. at 37°, in an attempt to induce the particles to aggregate or precipitate. The serum, however, set to a stable opaque gel, which when spread on a slide and examined under the ultramicroscope showed large gelatinous aggregates, interspersed with channels containing very small particles in very rapid motion, and in so great a concentration as to cause a hazy appearance from excessive reflexion. This gelation was reversed by restoration of *p*H to the normal value for serum, and the particle content also returned to normal; it was obviously a protein effect.

A set of tubes of centrifuged serum was then prepared, adjusted to *p*H values ranging from 3·0 to 4·6, with successive differences of 0·2 unit, and incubated for 24 hr., with examination at intervals for gelation. Table IV shows the results.

These results indicate that gelation occurs most rapidly at *p*H 3·6, which from Table III is a somewhat lower value than the point of maximum particle

Table IV. *Gelation of serum on incubation at varying pH*

	*p*H								
Hours	3·0	3·2	3·4	3·6	3·8	4·0	4·2	4·4	4·6
3	0	0	0	0	0	0	0	0	0
5½	0	0	0	TV	0	0	0	0	0
9	0	TV	G	G	AG	0	0	0	0
12	AG	AG	G	G	G	TV	0	0	0
24	AG	AG	G	G	G	TV	0	0	0

("G" = gelified, "AG" = almost gelified, "TV" = turbid and viscous.)

content. This does not suggest that the two effects are associated with each other; the gelation probably differs from the particle effect in being controlled by temperature as well as *p*H.

Behaviour of the particles in a high-speed centrifuge

We are very grateful to Prof. T. B. Davie and Dr J. D. A. Gray of the Department of Preventive Medicine, University of Bristol, for permitting us to make use of their "Ecco-Ultima" centrifuge in an endeavour to effect a separation of the particles from serum.

This machine was capable of a maximum speed of 15,000 r.p.m. Glass tubes were used for the preliminary experiments at medium speed, but they proved to be unable to withstand the highest speeds, being splintered under the strain. A set of duralumin tubes, supplied to fit the machine by Messrs A. Gallenkamp and Co., was found to withstand the strain of the maximum speed. The inner surfaces of the tubes on prolonged use became coated with a film of aluminium hydroxide, but this did not affect the results, and when rubbed up with 0·9 % NaCl gave an appearance under the ultramicroscope that was distinct from that of serum, the suspended particles settling rapidly.

The serum, previously centrifuged at low speed to remove suspended solid material, was usually centrifuged in the high-speed machine for a total period of 1 hr., but this was divided into three periods of 20 min. each, with 15-min. breaks to allow the machine to cool (the temperature was raised a few degrees at the high rate in spite of the cooling jacket).

After centrifuging for an hour, a small precipitate was always found at the bottom of the tube. Before pouring off the supernatant liquid, samples were taken from the surface ("top"), and from the middle and near the bottom of the tube by means of capillary pipettes, the corresponding samples from each of several tubes being mixed with one another. The supernatant liquid was then poured off, and generally sampled also after shaking. The precipitate from the tubes was suspended in 0·9 % NaCl for examination. Table V shows the results of a typical experiment.

The readings show that under the high centrifugal force the "LB" particles tend to move to the surface of the liquid, whilst the remaining particles move towards the bottom, where they ultimately pass out of suspension and collect as a precipitate. At the same time the general uniformity of the particle content of the serum shows that this separation is very gradual. The results suggest, however, that it should be possible to separate out the "LB" particles at very high speeds.

It may be mentioned here that many of our samples of serum, which were more fatty than usual, threw out a disc of white semi-solid material on the surface during their preliminary low-speed centrifuging. This when separated

Table V. *Fractions obtained from high-speed centrifuging of serum*

Serum centrifuged for 1 hr. at 15,000 r.p.m. Samples from top, middle and bottom of tube. Two slides made of each sample.

	Content	Size of particles	LB
Original serum	+ + +	(Mixed)	+
Top sample	+ +	(Mixed)	+ +
	+ +	(Mixed)	+ +
Middle sample	+ +	(Mixed)	−
	+ +	S—M	+
Bottom sample	+	M—S	−
	+	M—S	±
Decanted serum	+ +	(Mixed)	+
	+ +	(Mixed)	+
Precipitate in suspension	+ +	M	−
	+ +	(Mixed)	−

(The particle content of the suspension was increased by half-saturation with ammonium sulphate.)

and examined was found to be very rich in "LB" particles, whilst the bulk of the serum was comparatively poor. This material was extracted from the serum by ether, and all indications suggested that it was fat.

Behaviour of particles with proteolytic enzymes

Serum was digested with commercial preparations of pepsin, papain and pancreatin, and the effect on the particle content was followed. The enzyme preparations employed were the B.P. mixtures, glycerin. pepsin, glycerin. papain, and liq. pancreaticus. The pepsin and papain mixtures were diluted with 0·9 % NaCl before use. Each was adjusted to its optimum *p*H value. These mixtures when viewed under the ultramicroscope were found to be free of particles, or else to contain a few that settled rapidly on the slide.

The mixtures of serum and enzyme solutions were incubated in plugged sterile test tubes at 37°, and were examined in duplicate daily under the ultramicroscope. It was found that at least 3 days' incubation was required to produce any noticeable change.

Table VI shows the results of a typical experiment. Continuation of the incubation produced no further change. The total particle content was not reduced by any of the enzymes, whilst pancreatin alone caused a reduction in the "LB" particles. The experiments with pepsin presented considerable difficulty, owing to the ready precipitability of the serum proteins on the acid side of neutrality; the use of moderately concentrated solutions of pepsin resulted in turbid solutions after only 1 day's incubation. Conditions were also favourable for bacterial growth, which spoiled a number of experiments; the addition of preservatives was deemed inadvisable lest the physico-chemical state of the system should be excessively disturbed, whilst sterilization by heat was clearly impossible.

As a further experiment, the fractions obtained from one of the high-speed centrifuging experiments described earlier were incubated with pepsin and pancreatin similarly to the untreated serum. The glycerin. pepsin was replaced by a 0·5 % solution of pepsin in *N*/30 HCl; liq. pancreaticus was used as before. The top and bottom samples, and the precipitate suspended in 0·9 % NaCl, were all tested. Incubation was continued for 3–4 days. Results are shown in Table VII.

Table VI. *Serum and proteolytic enzymes*

Pepsin. Glycerin. pepsin, B.P., diluted 1 in 20 with 0·9% NaCl. Equal volumes of diluted solution and serum. *p*H 6·0.

Papain. Glycerin. papain, B.P., diluted 1 in 20 with 0·9% NaCl. Equal volumes of diluted solution and serum. *p*H 7·0.

Serum control mixture. Equal volumes of serum and 0·9% NaCl. Mixtures incubated at 37°.

	Content	Size of particles	LB
Serum control:			
Before incubation	+ +	(Mixed)	+ +
After 3 days' incubation	+ +	(Mixed)	+ +
Serum-pepsin:			
Before incubation	+ +	(Mixed)	+ + +
After 3 days' incubation	+ +	(Mixed)	+ +
Serum-papain:			
Before incubation	+ +	S	+
After 3 days' incubation	+	(Mixed)	+
	+ +	(Mixed)	+ + +

Pancreatin. Liq. pancreaticus, B.P., undiluted. 2 vol. to 1 vol. of serum. *p*H 8·0.

Serum control mixture. 2 vol. 0·9% NaCl to 1 vol. serum. Mixtures incubated at 37°.

	Content	Size of particles	LB
Serum control:			
Before incubation	+ +	(Mixed)	+
After 3 days' incubation	+ +	(Mixed)	+
Serum-pancreatin:			
Before incubation	+ +	(Mixed)	+
After 3 days' incubation	+ +	S—M	–
	+ +	(Mixed)	(+)
		(Settled rapidly)	
Pepsin solution	–		
Papain solution	–		
Pancreatin solution	–		

Table VII. *High-speed centrifuge and proteolytic enzymes*

Pepsin. 0·5% pepsin in *N*/30 HCl. Equal volumes of solution and serum. *p*H 6·0. Mixture incubated at 37°.

	Content	Size of particles	LB
Top sample:			
Before incubation	+	M—S	+
After 4 days' incubation	+ +	(Mixed)	+ +
Bottom sample:			
Before incubation	+	M	–
After 4 days' incubation	+ +	M	–
Precipitate in suspension:			
Before incubation	– }	(Particles settled)	
After 4 days' incubation	– }		

Pancreatin. Liquor pancreaticus, B.P., undiluted. 2 vol. to 1 vol. of serum. *p*H 8·0. Mixtures incubated at 37°.

	Content	Size of particles	LB
Top sample:			
Before incubation	+ +	L—M	+ +
	+	(Mixed)	+ +
After 4 days' incubation	+	(Mixed)	+
Bottom sample:	+ +	M—S	+
Before incubation	±	M—S	–
After 4 days' incubation	+ +	M—L	–
	+	M—S	–
	+	(Mixed)	–
		(Particles settled rapidly)	
Precipitate in suspension:			
Before incubation	+	M—S	–
After 3 days' incubation	±	S—M	–
Pepsin solution	–		
Pancreatin solution	–		

As was usual in the centrifuging experiments the "LB" particles were concentrated in the top sample. The concentration was noticeably reduced by pancreatin, but apparently unaffected by pepsin. "LB" particles were absent from the other samples, and the small and medium particles in all showed no significant change with either enzyme.

The disappearance of the "LB" particles on digestion with pancreatin is therefore the only noteworthy change caused by proteolytic enzyme preparations. Pancreatin contains lipase as well as trypsin, and its selective action on "LB" particles might well be explained by this fact, which would suggest that the "LB" particles are fat. The absence of any change in the small particle contents on digestion with proteolytic enzymes will be discussed in a later section.

The suspension of the precipitate from the high-speed centrifuge in 0·9% NaCl showed a slight reduction in its total particle content on digestion with pancreatin. This is to be expected on the hypothesis that the smaller particles are protein in nature, since the total amount of protein material present in such a suspension is necessarily limited. The precipitate also showed a reluctance to form a stable suspension in the acid pepsin mixture, the particles settling rapidly: this result when viewed together with the previous observations of precipitation of proteins and increase of particle content with fall of pH favours further the protein hypothesis.

Ultramicroscopic particles and filtration

The effect of filtration through paper and certain adsorbents on serum was studied in comparison with the effect of similar filtration on cow's milk, which when examined under the ultramicroscope shows a high content of particles of all sizes ranging from large fatty globules to particles similar to those observed in serum.

Milk. Milk was first filtered through a folded "Green's 500½" (agar) filter paper, and afterwards through a cone of calcium sulphate-carbonate mixture, prepared by moistening plaster of Paris, and after it had set powdering it in a mortar and adding 5% of precipitated chalk. The adsorbent was supported in an agar paper. Filtration was very slow, but sufficient filtrate was always obtained to permit the examination of all filtrates on the same day.

The filtrate from the agar paper was still white and opaque, but after passing through the $CaSO_4$—$CaCO_3$ mixture it appeared only opalescent.

A second portion of milk, after filtration through the agar paper, was filtered through a similar cone of "Kaylene" (colloidal kaolin). This also yielded an opalescent filtrate. Occasionally with both the adsorbents the filtration required repetition.

The optical examination of the milk and the various filtrates is recorded in Table VIII.

Table VIII. *Filtration of milk through adsorbents*

	Content	Size of particles	
Untreated milk	+ + +	M	Many large globules on slide. Film not uniform
Agar paper filtrate	+ + +	M	No large globules but numerous small globules and submicrons. Film uniform
$CaSO_4$—$CaCO_3$ filtrate	+ +	M	No globules
"Kaylene" filtrate	+ +	M—L	No globules

The particles observed in the milk show a noticeable difference from those in serum, being circular in appearance and steadier in brilliance. The optical appearance of the final milk filtrates was unaffected by centrifuging in the low-speed machine.

The two filtrates from the $CaSO_4$—$CaCO_3$ mixture and "Kaylene" filtrations were acidified with *N* HCl. A flocculent precipitate was produced that showed no Brownian movement; when this was removed by filtration or centrifuging a clear solution was obtained which was optically empty. The precipitate was insoluble in chloroform or ether, and when dried did not give the Salkowski reaction for cholesterol; it was most probably casein.

Serum. Serum was now subjected to filtration through agar paper and both adsorbents with exactly the same technique. Table IX shows the results obtained with two samples of serum filtered through "Kaylene", and one of them through $CaSO_4$—$CaCO_3$ mixture. Serum "A" was fresh, whilst serum "B" had been in cold storage for a week.

Table IX. *Filtration of serum through adsorbents*

		Unfiltered		Filtered	
Serum	Adsorbent	Content	Size of particles	Content	Size of particles
A	"Kaylene"	+ + (3 slides)	M	+ (3 slides)	(Mixed)
A	$CaSO_4$—$CaCO_3$	+ +	(Mixed)	+ +	(Mixed)
		+ +	S	+ +	(Mixed)
		+ +	S—M	+	S—L
B	"Kaylene"	+	(Mixed)	+	S
		+ +	(Mixed)	+	M
		+ +	(Mixed)	+	(Mixed)

It will be seen that filtration made very little difference to the particle content. Acidification of the filtrates resulted in a steady precipitation of serum proteins according to the amount of acid added; the precipitates on centrifuging off and drying were found to be free of cholesterol. The supernatant liquids were opalescent and heavily charged with particles.

Serum "B" showed an interesting result. Unlike many of our samples, it had not become contaminated with haemoglobin through haemolysis while separating from its blood clot. It was noticed that the yellow colour in the "Kaylene" filtrate had deepened almost to brown; in spite of this, the particle content showed no increase, but rather an apparent fall. This suggests that the lipochrome pigments of serum have no connexion with the ultramicroscopic particles. In view of the lack of effect of dilution of serum on the particle content, which will be discussed later, we cannot regard this result as absolute evidence of the retention of particles by kaolin. Serum "A", which contained haemoglobin, showed no colour change on filtration.

Lipins. For comparison, concentrated colloidal suspensions of cholesteryl oleate (B.D.H.) in soap solution, and of ovolecithin in distilled water, were filtered through agar paper and then through each adsorbent. The optical appearance of the suspensions before filtration showed distinct differences from that of serum. The cholesteryl oleate suspension showed circular, steadily-brilliant particles similar to those observed in milk, and also the same globules, some of which were small enough to pass through the agar paper. On the other hand, the lecithin particles were much less sharply defined than serum particles. Table X shows the effect of filtration on these suspensions.

Table X. *Filtration of lipin suspensions*

	Ovolecithin		Cholesteryl oleate	
Adsorbent	Content	Size of particles	Content	Size of particles
(Unfiltered suspension)	+ + +	M—S	+ +	(Mixed)
"Kaylene"	+ + +	S	+	(Mixed)
$CaSO_4$—$CaCO_3$	–	–	±	(Mixed)

The ovolecithin was adsorbed completely by the $CaSO_4$—$CaCO_3$ mixture, the filtrate being clear and colourless, and showing no Tyndall phenomenon—a direct contrast to the lack of effect on serum. The reduction of particle content in cholesteryl oleate suspensions by filtration through $CaSO_4$—$CaCO_3$ mixture was not regular in all our experiments, and the filtrate always showed a positive Tyndall effect.

Serum particles and extraction with organic solvents

The last group of experiments recorded in this paper was an investigation of the effect on the serum particles of extraction with the ordinary fat solvents.

Extraction was carried out by shaking the serum and solvent together in a mechanical shaker for periods of 6 hr.

Chloroform, carbon tetrachloride, benzene and light petroleum all caused denaturation of the serum proteins, generally precipitating them or forming a gel; the aqueous layer, examined when possible, contained particles in very large numbers, whilst the residues from evaporation of the organic solvent layers gave optically empty suspensions in 0·9 % NaCl. Ether was free of this disadvantage.

Serum was extracted three times with five times its volume of ether by the above technique. The aqueous layer after examination was evaporated to dryness and extracted again with ether in a Soxhlet apparatus, this second extract and the residue in the extraction thimble being weighed.

The ether extract from the serum, when evaporated to dryness, yielded an oily, evil-smelling residue, which was weighed and examined suspended in 0·9 % NaCl. The Salkowski test for cholesterol applied to this residue was positive.

The saponification values of the ether extracts of both the serum and the dried serum were determined to give an indication of their fat contents. Results are shown in Table XI.

Table XI. *Exhaustive extraction of serum with ether*

Serum (60 ml.) shaken three times with 5 vol. of ether for 6 hr.

	Content	Size of particles	LB
Original serum	+ + +	(Mixed)	+ + +
Ether-extracted serum	+ +	(Mixed)	+
Ether extract in 0·9 % NaCl	–		

	Weight of extract (mg.)	Saponification value
Ether extract of serum	127·6	130
Ether extract of dried serum	10·0	340

Weight of dried residue, 4·9 g.

It is seen from the table that practically the whole of the ether-soluble material was removed from the serum by the exhaustive treatment with ether. At the same time the "LB" particle content was very considerably reduced, but the total number of particles was not much less than before extraction.

Similar extractions with ether containing 6 and 12% of alcohol, which according to Mâcheboeuf & Sandor [1932] will remove the lipins completely from horse serum without affecting the proteins, caused considerable denaturation with pig serum, the aqueous layer becoming turbid and heavily charged with particles. Suspensions of the extracts in 0·9% NaCl were optically empty. The Salkowski reaction was positive, and lecithin also was identified in the extracts by hydrolysing with acid and formation of choline reineckate [Beattie, 1936].

Addition of alcohol to the serum followed rapidly by a large volume of ether also increased the number of particles by denaturing the proteins.

Table XII shows an experiment similar to that of Table XI, carried out on human serum from a case of paratyphoid fever. This serum was comparatively poor in "LB" particles at the outset. Results otherwise resemble those in Table XI.

Table XII. *Extraction of human (paratyphoid) serum with ether*

Serum (10 ml.) shaken three times with 5 vol. of ether for 6 hr.

	Content	Size of particles	LB
Original serum	+ +	M—S	+
Ether-extracted serum	+ +	M—S	±
Ether extract in 0·9% NaCl	−		

	Weight of serum (mg.)	Saponification value
Ether extract of serum	36·8	160
Ether extract of dried serum	4·6	50

Weight of dried serum, 580·6 mg.

In the next experiment three separate volumes of pig serum were extracted once with the same five volumes of ether to obtain a large quantity of ether-soluble material. The ether was partly taken up into the serum layers during the second and third extractions, forming a viscous mixture which, when allowed to stand in the refrigerator in a separating funnel, separated slowly into three layers, a "middle layer" of colourless, viscous liquid being formed between the usual serum and ether layers. The course of the separation of the ether and "middle layer" was followed in the ultramicroscope.

The ether extract, when evaporated down, yielded a moderately large amount of solid material, whilst Soxhlet extraction of the dried serum and "middle layer" with ether yielded relatively small quantities, that from the dried serum being negligible. The saponification values of all three extracts were determined.

The total content of lipin material of the ether extract of the serum was determined roughly by extracting a weighed quantity of the solid material with light petroleum and weighing the extract. About 90% of the material was dissolved.

We are indebted to Prof. E. L. Hirst and his staff for kindly carrying out a micro-determination of the nitrogen content of the material.

It will be seen from Table XIII that a large number of "LB" particles were left in the serum mixture after shaking with ether was completed, but that on standing these all passed into the "middle layer". The latter after drying contained a considerable percentage of ether-soluble material, but the dried serum layer was practically free of it. The ether extract of the serum was apparently all fat except for 10% (about 5% being protein from the nitrogen figure), yet the total serum particle content was unchanged by extraction with ether. The

Table XIII. *Extraction of serum with restricted quantities of ether*

3 vol. serum, 60 ml. each, extracted for 6 hr. with the same 300 ml. ether

	Content	Size of particles	LB
Original serum	+ +	(Mixed)	+ + +
Ether extract in 0·9% NaCl	–		
Unseparated serum mixture	+ +	(Mixed)	+ + +
Half-separated serum layer	+ +	(Mixed)	+ +
Completely separated serum layer	+ +	(Mixed)	+
"Middle layer"	+ +	(Mixed)	+ + +

		Ether extracts	
	Serum	Dried "middle layer"	Dried serum layer
Weight of dried material	—	566 mg.	3800 mg.
Weight of extract	242·6 mg.	20·2 mg.	12·2 mg.
% of dried material extracted	—	3·57%	0·09%
Saponification value	120	171	160
Iodine value	75	8	6
Nitrogen	0·4%	—	—
% of material soluble in light petroleum	90%	—	—

relatively large quantity of ether-soluble material in the "middle layer" as compared with the serum layer, and its high saponification value and low iodine value, suggest that the "LB" particles that passed into the "middle layer" were composed of saturated neutral fat.

The absence of any effect on the number or size of the particles in serum by treating with equal volumes of sodium tauroglycocholate solutions in concentrations up to 10% showed that the particles were not free fatty acid. They also were not precipitated by ricin, which showed that they were not cholesterol.

Extraction after high-speed centrifuging. Extraction three times with ether of a sample of serum that had been centrifuged for an hour in the "Ecco-Ultima" machine at 15,000 r.p.m. yielded the same amount of fatty material as did extraction of an equal volume of uncentrifuged serum. The precipitate which separated out gave no indication of being soluble in ether, whilst on the other hand, half-saturation with ammonium sulphate of its suspension in 0·9%

Table XIV. *Extraction of milk filtrates*

"Kaylene" and $CaSO_4$—$CaCO_3$ filtrates, 30 ml. Extracted three times for 6 hr. with 150 ml. ether

	Content	Size of particles
"Kaylene" filtrate	+ + +	M
Ether-extracted "Kaylene" filtrate	+ + + +	M
$CaSO_4$—$CaCO_3$ filtrate	+ + +	M
Ether-extracted $CaSO_4$—$CaCO_3$ filtrate	+ + + +	M

	Weight of material	Saponification value
"Kaylene" filtrate:		
Ether extract	19·0 mg.	301
Ether extract of dried residue	8·4 mg.	—
$CaSO_4$—$CaCO_3$ filtrate:		
Ether extract	4·0 mg.	670
Ether extract of dried residue	7·2 mg.	—
Weights of dried residues:		
"Kaylene"	2·3 g.	—
$CaSO_4$—$CaCO_3$	2·4 g.	—

NaCl increased considerably its particle content, suggesting a protein origin for the particles.

Milk filtrates. Table XIV shows the figures obtained by extracting the "Kaylene" and $CaSO_4$—$CaCO_3$ filtrates of milk in a similar way to serum. The amount of fat obtained in each case was negligible, but the particle content, already large, showed a small increase.

These results support the view that both milk and serum particles are protein in nature.

Sera from different species

A few comparative experiments were made with pig, sheep and cow sera. Sheep serum resembled pig serum both externally and ultramicroscopically; cow serum, on the other hand, was coloured an intense yellow by lipochrome pigments. The optical pictures (Table XV) show no great differences.

Table XV. *Comparison of sera from different species*

Serum	Content	Size of particles	LB
Pig	+ +	M—S	+
Sheep	+ +	M—S	+
Cow	+	S—M	−

Sheep and cow sera were extracted three times with five volumes of ether as for pig serum. The sheep serum was unaffected by the extraction, but a considerable quantity of the yellow pigment was removed from the cow serum; the concentrations in the unextracted and extracted sera as compared in a Hellige colorimeter were in the ratio 9 : 5. The ether extracts were evaporated and weighed. Table XVI shows the optical results and the weights of the extracts.

Table XVI. *Extraction of sheep and cow sera*

30 ml. each serum, extracted three times for 6 hr. with 150 ml. ether

	Content	Size of particles	LB
Sheep serum	+ +	S—M	+
Ether-extracted sheep serum	+ +	S—M	−
Cow serum	+ +	(Mixed)	+
Ether-extracted cow serum	+ +	(Mixed)	−

Weights of extracts: Sheep serum, 40 mg. Cow serum, 42·8 mg.

The extract from cow serum was soluble in light petroleum except for about 6·6 %.

These figures show that the behaviour of the particles in sheep and cow sera with ether is essentially the same as that of pig serum particles. The removal of the pigment from cow serum has no visible effect on its particle content.

Discussion

We have indicated after each of our lines of investigation the conclusions we have reached from the results therefrom as to the chemical nature of the ultramicroscopic particles in serum. Certain of these and points arising therefrom require further discussion.

With regard to the smaller particles, practically all the evidence shown supports McDonagh's conclusion that they are protein in nature. They may consist of both globulin and albumin; the results with half-saturation with ammonium sulphate show in fact that both can produce particles. The small maximum shown in the particle content when serum is brought to *p*H 4·0–4·2

probably represents an isoelectric point for one particlular protein. In connexion with this conclusion as to the smaller particles we are greatly indebted to Dr L. F. Hewitt for the gift of a sample of his "fat-free" horse serum [Hewitt, 1927], which when viewed under the ultramicroscope gave the reading "+ + M—S". The experiments with sera from different species recorded in this paper indicate that there is no essential difference between the small particle contents of any particular species, so that the above reading can be taken as typical for fat-free normal sera.

It is interesting to consider the effects of diluting the serum, which contrary to expectation does not appear to cause any considerable reduction in its small particle content; the particles are obviously being reinforced from some source within the serum. The ether extraction results make it highly improbable that the serum lipins are the source concerned so that the extra particles must therefore be drawn from the serum proteins. This does not at first appear to be an acceptable conclusion, since dilution would be expected to facilitate hydration of the proteins. We must postulate an "equilibrium concentration" of unhydrated protein in every serum, which always tends to re-establish itself on dilution, and possibly also on concentration, of the serum. Such an equilibrium concentration may probably be of considerable physiological and pathological importance. It represents in undiluted normal serum only a very small fraction of the total serum proteins, on which it is not completely dependent. It should explain the apparent lack of effect of the proteolytic enzymes on the particles, since any unhydrated protein that was digested would immediately be replaced by dehydration of fresh protein. The reduction by pancreatin of the particle content of the suspension of the precipitate from high-speed centrifuging is to be expected on this hypothesis, for the suspension contains no reserve of hydrated protein to replace digested particles.

The large bright particles tend to "follow the fat" in every process to which the serum has been subjected, and are most probably neutral fat, which may be saturated or unsaturated.

Frazer & Stewart [1937] reported that the number of particles of all sizes in normal human serum was increased by ingestion of fat, but apparently unaffected by protein. They concluded from these observations that the particles were of lipoid origin. Our results do not support this conclusion, except for the "LB" particles. We suggest that the absorption of the extra fat into the blood causes changes in the colloidal state of the serum, which lead to an increase in the equilibrium concentration already postulated of the unhydrated protein, which recovers its normal value on removal of the fat. Protein passing into the blood does not affect the colloidal picture in this way, since the equilibrium concentration, as stated above is governed principally by physical factors, the absolute concentration of the serum proteins being only indirectly concerned if at all. The equilibrium concentration hypothesis should also explain Frazer & Stewart's observation that the particles in normal serum are maintained at a constant level in starvation.

One of us [Peters, 1936] has recorded the examination of serum from a case of acute tuberculosis. The picture was heavily charged with particles and aggregates of gelatinous material, and was almost identical with that which we obtained with serum gelified by incubation at pH 4·2–4·4. Since serum globulin is known to increase largely in tuberculosis, we conclude from this observation that the clotting of fibrin in tuberculous blood leaves the serum so highly concentrated that the equilibrium concentration effect is overcome, and the globulin is dehydrated in large quantities and gelified.

SUMMARY

1. The ultramicroscopic particles in serum are of three distinct sizes: large bright ("LB"), medium-dull, and small dull.

2. The number of particles is not diminished by dilution up to three times the volume of the serum with 0·9 % NaCl.

3. The particles are not appreciably affected by half-saturation of the serum with ammonium sulphate, although some particles are to be seen when the globulin precipitate so obtained is suspended in 0·9 % NaCl. Complete saturation removes the particles with the proteins.

4. Variation of *p*H towards the alkaline side causes disappearance of the particles. On the acid side the particles increase with the acidity until the proteins begin to precipitate. A small maximum occurs at *p*H 4·2–4·4.

5. The particles are vastly increased in number when serum is gelified by incubation at *p*H 3·6. Restoration of *p*H to normal also restores to normal the particle content.

6. When serum is centrifuged at 15,000 r.p.m., the "LB" particles move to the surface of the liquid, but the smaller particles appear to be drawn to the bottom.

7. The smaller particles in serum are apparently unaffected by proteolytic enzymes, but the "LB" particles are removed by pancreatin, probably through the action of lipase.

8. Filtration of milk through colloidal kaolin or $CaSO_4$—$CaCO_3$ mixture removes all the fat, but leaves a suspension of ultramicroscopic particles resembling those of serum. Serum particles are scarcely affected by filtration through either adsorbent. Lipins are either partly or wholly removed from suspension by $CaSO_4$—$CaCO_3$ mixture.

9. Prolonged extraction of serum with several changes of ether removes all the fat, and the "LB" particles disappear at the same time, but the number of the smaller particles is unaffected. Extraction of several charges of serum with the same ether results in formation of a "middle layer" which contains some saturated neutral fat, and also a large number of "LB" particles.

10. The sera of pigs, sheep and cows, and human serum from a case of paratyphoid fever, show no essential difference in their particle contents nor their behaviour with ether. The lipochrome pigments in cow serum are extracted by ether without affecting the particle content.

11. It is concluded that the smaller particles are protein as originally stated by McDonagh [1927], but that the "LB" particles are neutral fat.

12. An "equilibrium concentration" of unhydrated protein in serum is postulated, which is governed partly or wholly by physical factors and tends to re-establish itself on dilution and possibly concentration of the serum.

We are greatly indebted to the Sir Halley Stewart Trust for grants which have made this work possible.

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CXCVII. THE VITAMIN A_1 AND A_2 CONTENTS OF MAMMALIAN AND OTHER ANIMAL LIVERS

By ALBERT EDWARD GILLAM

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(*Received 21 July 1938*)

THE liver fats of animals in general rank amongst the richest sources of the growth-promoting and anti-xerophthalmic substance known as vitamin A which, in addition to its biological activity, is characterized by an ultra-violet absorption band near 328 mμ and by the blue colour that it gives with antimony trichloride in chloroform (main absorption maximum at 620 mμ). These properties of the vitamin are shown with slight modification by the liver fats both of marine fish and of mammals. When, however, the first preparations of really potent concentrates of the vitamin were obtained [Karrer *et al.* 1931; Heilbron *et al.* 1932] they were obviously heterogeneous, notably because they contained small but variable quantities of a substance exhibiting an absorption band at 693 mμ in the $SbCl_3$ blue colour [Heilbron *et al.* 1932]. It has since been observed that the liver fats of certain Russian freshwater fish contain more of the 693 mμ chromogen than of the more common one characterized by a 620 mμ band [Lederer & Rosanova, 1937] and more detailed spectrophotometric examinations of the liver oils of a variety of freshwater fish [Lederer *et al.* 1937; Edisbury *et al.* 1937; Gillam *et al.* 1938; Edisbury *et al.* 1938] have confirmed this. The same investigations have also shown that the ultra-violet absorption spectra of the oils are characterized by two maxima near 280 and 350 mμ, respectively, instead of the single maximum at 328 mμ common to the liver oils of marine fish.

It has been suggested that the 693 mμ chromogen might be a second vitamin A, possibly specific to freshwater fish [Lederer *et al.* 1937], whilst Edisbury *et al.* have tentatively named it vitamin A_2 [1937] and later "factor A_2" [1938]. Although in our earlier concentration of the substance it was not found possible to separate it completely from vitamin A_1 the results of feeding experiments on rats [Gillam *et al.* 1938] indicate that it actually possesses the biological activity always associated with vitamin A_1, whilst the chemical evidence suggests that it is probably a higher isologue ($C_{22}H_{32}O$) of this vitamin which, itself, has been shown to have the formula $C_{20}H_{30}O$ [Karrer *et al.* 1931; Heilbron *et al.* 1932]. More recent experiments with the physiologically potent concentrate previously examined have shown that it contains another chromogen (645–650 mμ) the presence of which makes the 620 mμ chromogen value too high [Lederer, 1938]. On the assumption that this chromogen has no vitamin A activity itself, Lederer [1938] draws the conclusion that more of the total activity of the concentrate must be due to the 693 mμ chromogen than was previously supposed. This further justifies the title vitamin A_2, as also does the fact found by Wald [1937, 1, 2] that the 693 mμ chromogen can replace vitamin A_1 in the visual cycle that occurs in the retinae of freshwater fish.

The generalization that is beginning to emerge from these results is that whereas marine fish liver oils contain the 620 mμ chromogen (vitamin A or A_1) with only traces of the 693 mμ chromogen (vitamin A_2),[1] freshwater fish liver oils

tend to contain more vitamin A_2 than vitamin A_1, or at least a larger proportion of vitamin A_2 than do the marine fish liver oils. The only mammals that have been examined for vitamin A_2, namely, the rabbit [Edisbury *et al.* 1938] and the whale (private communication from Dr R. A. Morton), apparently contain none of this substance.

The present paper records the results obtained by the examination of the livers of a series of animals and birds with particular reference to the total vitamin A content of the liver and the presence or absence of vitamin A_2.

Experimental

The procedure adopted in all cases was, firstly, hot saponification of the fresh liver with KOH (2 ml. 10% KOH in 50% aqueous alcohol per g. of fresh liver: time of saponification 1–3 hr.) until the tissue was completely broken down. After cooling, the mixture was diluted with water and extracted with ether, the resulting extract being washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness in N_2. Part of the unsaponifiable matter so obtained was treated with $SbCl_3$ in chloroform, the blue colour being examined spectroscopically by methods already described [Heilbron *et al.* 1931]. Values for the relative amounts of vitamins A_1 and A_2 present were obtained from the ratio of the intensities of the absorption bands at 620 and 693 mμ, respectively, of the blue solution. The remaining unsaponifiable matter in chloroform was diluted 10–20 times with alcohol and its ultra-violet absorption spectrum determined photographically, using a Hilger E3 quartz spectrograph. The vitamin A content was taken as the mean of the two values obtained from the intensity of absorption of the alcoholic and chloroformic $SbCl_3$ solutions, respectively, on the provisional basis for pure vitamin A of $E^{1\%}_{1\text{ cm.}}{}_{328\,m\mu=1600\,(\text{alcohol})}$ [Carr & Jewell, 1933].

The results are recorded in Table I.

Discussion

Consideration of the data and of previous work [cf. Kuhn, 1933] leads to the conclusion that in marine fish in general (or possibly in some of the animals on which they feed) carotene is ingested (either directly or indirectly from the phytoplankton) and the $C_{40}H_{56}$ molecule is broken down into C_{20} fragments constituting the typical vitamin A. The small amount of vitamin A_2 normally present would appear either to be produced as a by-product of this process in the fish themselves, or to be received by them preformed to this small extent from the lower animals on which they feed. On the other hand, freshwater fish (or again some lower organisms eaten by them) must be able, in addition, to break down the carotene molecule into C_{22} and C_{18} fragments, the C_{22} fragment, at least, appearing in the liver in greater concentration than is normally met with in marine fish livers.

The complete absence of vitamin A_2 from the livers of the majority of both the individuals and the species of mammals and birds examined indicates that

[1] Karrer has suggested the name "Axerophthol" for the classical vitamin A and the class name "Axerophthols" for the higher isologues [Euler *et al.* 1938]. Using Karrer's nomenclature, the particular axerophthol ($C_{22}H_{32}O$) which our earlier experiments indicate as the 693 mμ chromogen would be called *β-apo*-5-carotinol. Karrer and his collaborators have prepared what they believe to be this compound and whilst it gives a blue colour with $SbCl_3$ the position of the absorption band that gives rise to the colour is apparently quite different from 693 mμ. If the formula of the compound is beyond doubt then the formula suggested for the 693 mμ chromogen will need modification.

Table I

Animal	Source, etc.	Wt. fresh liver g.	Estimated total vitamin A mg.	Vitamin A mg. per 100 g. liver	693 mμ chromogen	Ratio of intensities 693/620 mμ
Lion *Felis leo*	Zoological Gardens (20 yr. old)	2000	160	8·0	Absent	0·057
Gibbon *Hylobates concolor*	Zoo (6 months old)	110	0·75	0·70	,,	Not measurable
Giant monitor *Varanus salvator*	Zoo	(*a*) 200 (*b*) 113	500 74	250 65	Present —	0·11 —
Python *Python reticulatus*	Zoo (very old animal)	800	688	86	Absent	0·023
Alligator *Alligator mississippiensis*		225	8·0	3·5	,,	<0·20
Rabbit *Lepus cuniculus*	Wild	27·6	4·75	17·2	,,	0·073
Rat *Mus decumanus*	Wild town animal, ♂	9·2	0·55	6·0	,,	0·031
Mouse *Mus musculus*	Laboratory animals (*a*) Av. of 9 (*b*) Av. of 15	 1·63 1·14	 0·013 0·0125	 1·1 1·1	 ,, ,,	 Not measurable —
Guinea-pig *Cavia porcellus*	Laboratory animals Av. of 3	10·4	0·07	0·68	,,	0·076
Dog		278	3·06	1·1	,,	<0·20
Cat		66·5	2·4	3·6	,,	0·033
Ox		5235	340	6·5	,,	0·052
Cow		6075	132	2·17	,,	0·082
Lamb		(*a*) 506 (*b*) 280	56 51	11·0 14·7	,, ,,	0·058 0·063
Pig		(*a*) 844 (*b*) 1060	67 10	7·94 1·0	,, ,,	0·044 —
Frog *Rana temporaria*	Local, av. of 6	0·41	Order of 0·0043	1·04	,,	Not significant
White Whale *Beluga leucas*		—	—	—	,,	0·066
Hen	Av. of 2	28	3·4	12·1	,,	0·068
Duck	Av. of 4	24	2·93	12·2	,,	<0·14
Turkey		(*a*) 118 (*b*) 128	24·7 26·4	21·0 20·7	,, ,,	0·044 0·06
Pigeon	Av. of two young birds	4·2	1·37	32·5	,,	0·10
Kingfisher *Alcedo ispida*	Cheshire (one young fledgeling: found dead)	1·9	0·15	7·7	Not detected	—
Seal *Phoca vitulina*	Russia	—	—	—	Present	0·20
Otter *Lutra vulgaris*	Cumberland	191	90	47	,,	0·22
Rat (fed on vitamin A_2 concentrate)		—	—	—	,,	1·40

they cannot convert carotene directly into vitamin A_2 for, in the herbivorous animals at least, there can be no doubt that the diet normally contains relatively large quantities of carotene. The question arises, however, whether mammals can actually pass preformed vitamin A_2 into the liver when it is taken into the digestive tract. To test this point Dr Lederer very kindly supplied us with extracts of the livers of rats fed on concentrates of vitamin A_2. Examination of these extracts (cf. Table I) revealed the presence of relatively large quantities of the 693 mμ chromogen; in fact the livers of the rats were, chromogenically, very similar to those of freshwater fish.

Since mammals normally do not appear to manufacture the 693 mμ chromogen but can pass it into the liver when it is contained in the food eaten, it was argued that mammals that feed on fish should store some of this substance in their livers. Interesting test cases of this point are provided by the seal, for a liver extract of which we are indebted to Dr Lederer, and the otter, of the liver of which a sample was obtained from Cumberland through the kindness of Dr W. S. M. Grieve. Examination showed that both these livers contained vitamin A_2 in easily detectable amounts (cf. Table I). On the other hand the only specimen of a bird feeding on freshwater fish that we could obtain (i.e. the kingfisher) was found to have no detectable vitamin A_2 in its liver[1] (cf. Table I). Thus of all the mammals examined the only ones having the 693 mμ chromogen present in the liver were those known to feed on, or to have fed upon, fish. Of the remaining animals and birds examined the only other having any detectable quantity of this chromogen was the giant monitor in which, however, the proportion of this substance present was very small (cf. Table I).

It is interesting to note that the python and the giant monitor had the largest total weight of vitamin A_1 per liver of all the species examined; moreover, on the basis of percentage weight of vitamin in the liver, these two species stand alone. The particular python examined was a very old 25 ft. specimen and it may be that, as in the case of the halibut where the vitamin content of the liver increases with age, individuals of this species also accumulate vitamin A as they become older. Of the remaining species examined with the exception of the otter the percentage of vitamin A in the liver only varied within relatively narrow limits; thus in 18 species the average was 8·3 mg. per 100 g. fresh liver, whilst the limits of variation were of the order 1 to 32 mg. per 100 g. When mammals are compared with fishes much larger variations are, however, observed [cf. Edisbury *et al.* 1938].

In this preliminary survey only a few individuals of each species have been examined but it is probable that if more were examined much wider variations would be found. On the other hand the work of Davies & Moore [1935] indicates that the level of vitamin A in the liver of the rat usually falls within narrow limits; these workers have shown that when rats are fed with very high dosages of vitamin A the abnormally high resultant value for the vitamin content of the liver rapidly falls to normal as soon as the vitamin intake is reduced.

The differences between freshwater fish, marine fish, mammals and birds in the reaction of their liver extracts towards $SbCl_3$ are summarized in Table II.

Table II. *Summarized data on the relative amounts of 693 and 620 mμ chromogens in the livers of fish, animals and birds*

	No. of species included*	Ratio of the intensities of absorption at 693/620 mμ		
		Range found	Average	693 mμ absorption band
Freshwater fish†	11	0·25–2·62	1·680	Usually strongly positive
Marine fish†	11	0·04–0·17	0·110	Normally just detectable
Animals (almost all mammals)	11	0·03–0·17	0·060‡	Only rarely present
Birds	5	0·04–0·10	0·068‡	Only rarely present

* Some of these species have been represented by many specimens, others by only one.

† Cf. Gillam *et al.* [1938] and Lederer [1938] for some detailed examples.

‡ These values, which might be expected to be zero in the absence of the 693 mμ band, probably represent the overlap of the 693 mμ band at 620 mμ.

[1] The author is indebted to Miss Legge of the Manchester Museum for this specimen.

SUMMARY

Extracts of the livers of a number of species of animals and birds have been examined spectroscopically with particular reference to their contents of vitamins A_1 and A_2.

It has been found that, in general, mammal and bird livers contain vitamin A_1 but no vitamin A_2. In the 21 species examined the only exceptions found were the giant monitor (considerable vitamin A_1 and only traces of vitamin A_2) and animals known to feed on fish, i.e. the seal and the otter. A mammalian liver exceptional in that it contained more vitamin A_2 than vitamin A_1 was obtained by feeding a rat with a concentrate of freshwater fish liver. It would thus appear that although mammals cannot metabolize vitamin A_2 they can pass it into the liver if they receive it preformed into their digestive tract.

The vitamin A_1 contents of all the livers examined fell within the rather narrow limits of 0·7–32 mg. per 100 g. fresh liver (average for 18 species = 8·3 mg. per 100 g.) with the exception only of the python, giant monitor and otter with values of 86, 250 and 47 respectively.

The author is indebted to Prof. I. M. Heilbron for his interest in this work and to Dr J. F. Wilkinson and Mr G. Iles, Curator of the Belle Vue Zoological Gardens, Manchester, for specimens of the livers of certain animals. He also owes thanks to Dr R. W. Fairbrother, Dr T. H. B. Bedford and Dr W. Schlapp of this University for specimens of other animals' livers.

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CXCVIII. VITAMIN B_1 AND COCARBOXYLASE IN ANIMAL TISSUES[1]

BY SEVERO OCHOA AND RUDOLPH ALBERT PETERS

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(*Received 23 July 1938*)

THE discovery by Lohmann & Schuster [1937] that cocarboxylase is the pyrophosphoric ester of vitamin B_1 suggested that in animal tissues the vitamin is active like this ester in the oxidation of pyruvic acid. It now seems clear that animal tissues and certain bacteria do not decarboxylate pyruvic acid to acetaldehyde and CO_2 (as yeast does) but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions or directly [Krebs & Johnson, 1937; Lipmann, 1937, 1; Weil-Malherbe, 1937]. Lipmann [1937, 2] reports that with acetone preparations from *B. Delbrückii* the simultaneous decarboxylation and oxidation of pyruvic acid require cocarboxylase.

In animal tissues evidence that vitamin B_1 pyrophosphate is concerned in the oxidation of pyruvic acid is derived (1) from catatorulin tests, and (2) from the presence of cocarboxylase in tissues and their alleged capacity to synthesize cocarboxylase from vitamin B_1. The first point, that cocarboxylase can replace vitamin B_1 in catatorulin tests [Lohmann & Schuster, 1937], has not been confirmed in this laboratory using "teased" brain [Peters, 1937]; more recent unpublished experiments with brain slices also gave negative results. This point obviously requires further elucidation.

In regard to the second point, Auhagen [1932] first showed that boiled extracts of animal tissues stimulated the decarboxylation of pyruvic acid by yeast preparations (aetiozymase), indicating the presence of cocarboxylase. Simola [1932] investigated the influence of nutrition upon this phenomenon. Synthesis of cocarboxylase from vitamin B_1 by minced animal tissues or various tissue preparations has been reported from several laboratories [von Euler & Vestin, 1937; Tauber, 1937; Lohmann & Schuster, 1937; Peters, 1937], whereas Stern & Hofer [1937] reported negative results. This work is not yet sufficiently quantitative and further it has not so far been ascertained whether vitamin B_1 is present in tissues in the free form.

We have now developed a method which allows the separate quantitative estimation of cocarboxylase and free vitamin B_1, by means of which the following points have been investigated: (1) the cocarboxylase and vitamin B_1 contents of boiled extracts from normal and avitaminous tissues, (2) the enzymic synthesis of cocarboxylase from vitamin B_1. In the present paper we shall show that there is much more cocarboxylase than vitamin B_1 present in animal tissues, and that it is much reduced when vitamin B_1 is withheld from the diet, and further, that the liver readily synthesizes cocarboxylase from vitamin B_1 *in vivo*. Another paper will deal with the synthesis of cocarboxylase *in vitro*. Various organs (brain, muscle) have only a very limited power of synthesis;[2] intestinal mucosa does not show any activity at all, whereas active preparations can be obtained from the liver.

[1] A preliminary report of this work appeared in the *Trans. Soc. Chem. Ind.* 57, 470, 1938.

[2] Less in the case of brain than suggested by Peters [1937], who did not know of the stimulating effect of vitamin B_1 upon the action of cocarboxylase (cf. below).

Experimental

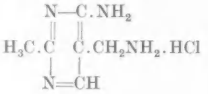
Dry baker's yeast (supplied by Distillers Co. Ltd.) has been used throughout. It was freed from cocarboxylase by rapid washing with $M/10$ Na_2HPO_4 (1 g. yeast 3 times with 50 ml. each time) and once with distilled water at room temperature.[1] The washed yeast was suspended in 10 ml. $M/10$ phosphate buffer of pH 6·2. The production of CO_2 from pyruvic acid was measured manometrically using both Barcroft and Warburg manometers. Each bottle contained 1·0 ml. washed yeast suspension (added last), 0·1 ml. $MgCl_2$ (0·1 mg. Mg), 0·4 ml. water or experimental solutions and 0·2 ml. sodium pyruvate adjusted to pH 6·2 (5 mg. pyruvic acid). The reaction was started after 10–12 min. by tipping in the pyruvate solution from the side bulb or dangling tube. The total volume of fluid was 1·7 ml., the temp. was 28° and the gas either air or nitrogen.

Preparations

Cocarboxylase. One pure specimen from Dr K. Lohmann (Berlin); another specimen (70 % purity, vitamin B_1-free) prepared by Mr H. W. Kinnersley from yeast crude cocarboxylase, obtained by enzymic synthesis from synthetic vitamin B_1 by the method of Kinnersley & Peters [1938].

Vitamin B_1 chloride hydrochloride. Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

Pyrimidines and thiazole vitamin components. From Prof. A. R. Todd (Lister Institute, London).



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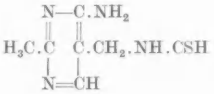

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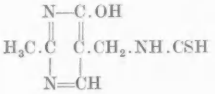
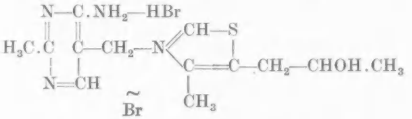


III

Vitamin B_1 analogue from 4-methyl-5-β-hydroxypropylthiazole (referred to in Table II as vit. B_1 analogue IV) from Dr Buchman (Pasadena).

IV 

Cozymase. Prepared from yeast by the method of Meyerhof & Ohlmeyer [1937] by Mr L. A. Stocken in this laboratory; this was 91 % pure by comparison with pure cozymase kindly supplied by Dr Ohlmeyer. Other compounds used came from commercial sources.

We are much indebted to those mentioned above for their kind gifts which have made this work possible.

1. *The activation of cocarboxylase action by vitamin B_1*

Whereas vitamin B_1 cannot replace cocarboxylase, it has been found by one of us [Ochoa, 1938] that it markedly stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase. The effect is apparent with 0·1γ and increases with increasing concentrations of the vitamin;

[1] The above method of washing was adopted when Lohmann's method failed. At first the latter gave good results.

with 1·0γ cocarboxylase it is nearly maximal for 15γ vitamin B_1 hydrochloride. Fig. 1 illustrates this. The activation is the same in air as in N_2 showing that it is not due to oxidative removal of acetaldehyde. That the vitamin does not induce a disappearance of acetaldehyde by dismutation is shown by the fact

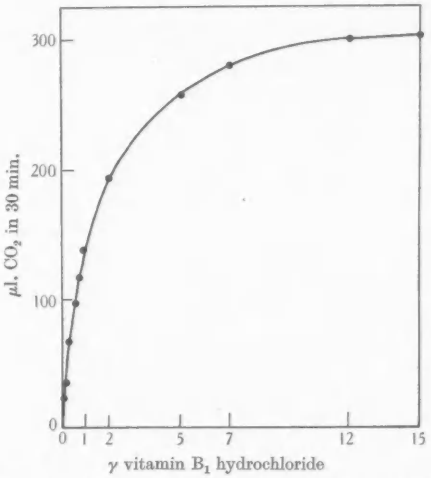


Fig. 1. Activation of cocarboxylase action by vitamin B_1. Ordinate μl. CO_2 evolved in presence of increasing amounts of vitamin and 1γ cocarboxylase, minus CO_2 evolved with 1γ cocarboxylase alone (1γ cocarboxylase = 163 μl. CO_2; control = 26 μl. CO_2 in 30 min.).

that no acid production takes place (measured in bicarbonate solution; gas, pure CO_2). Further, iodoacetic acid, while depressing the decarboxylation to some extent, has practically no effect on the activation by the vitamin, as shown in Table I.

Table I. *Action of iodoacetic acid on the stimulation of cocarboxylase by vitamin B_1*

		μl. CO_2 in excess of control in 30 min.			
Exp. no.	Molarity of IAA	1γ cocarb.	1γ cocarb. + 10γ vit. B_1	Activation by vit. B_1	% inhibition of vitamin activation
1	0	211	505	294	—
	0·0009	187	479	292	0
2	0	162	465	303	—
	0·0050	115	373	258	15

The stimulating effect of the vitamin is due to the pyrimidine part of the molecule and is absent in the absence of the NH_2 group. A number of other compounds investigated were found to be inactive (Table II).

Vitamin B_1 monophosphate has no cocarboxylase activity (as already stated by Lohmann & Schuster), whether alone or together with vitamin B_1, but it was found to stimulate cocarboxylase action. The monophosphate was obtained by acid hydrolysis of cocarboxylase (15 min. in *N* HCl at 100°); the solution was used for the experiments after bringing the reaction to *p*H 6·2 with NaOH (Table III).

Table II. *Effect of various compounds on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase*

Exp. no.	Sample	μl. CO_2 evolved in 30 min.
1	Control	15
	1γ cocarboxylase	160
	1γ cocarb. + 2γ vitamin B_1	344
	1γ cocarb. + 20γ pyrimidine I	385
	1γ cocarb. + 50γ pyrimidine II	318
	1γ cocarb. + 90γ pyrimidine II	385
	1γ cocarb. + 90γ pyrimidine III	161
	1γ cocarb. + 20γ vitamin B_1 analogue IV	432
	1γ cocarb. + 90γ thiazole	150
	1γ cocarb. + 100γ cytidylic acid	168
	1γ cocarb. + 100γ vicine	158
	1γ cocarb. + 100γ adenosine	160
	1γ cocarb. + 100γ nicotinamide	153
2	1γ cocarboxylase	178
	1γ cocarb. + 50γ acetylcholine	174
	1γ cocarb. + 100γ choline	161
	1γ cocarb. + 100γ spermine	154

Table III. *Effect of vitamin B_1 monophosphate on the decarboxylation of pyruvic acid by alkaline washed yeast with and without addition of cocarboxylase. (Quantities expressed for vitamin B_1 monophosphate as γ cocarboxylase; 1γ cocarboxylase = 0·6γ vitamin B_1)*

Sample	μl. CO_2 evolved in 30 min.
Control	9
10γ vitamin B_1	14
10γ vitamin B_1 monophosphate	12
10γ vitamin B_1 + 10γ vitamin B_1 monophosphate	14
1γ cocarboxylase	135
1γ cocarb. + 1γ vitamin B_1 monophosphate	188
1γ cocarb. + 2γ ,,	203
1γ cocarb. + 5γ ,,	257
1γ cocarb. + 10γ ,,	315

Recently Lipschitz *et al.* [1938], observing that addition of vitamin B_1 to alkaline washed yeast containing Mg^{++}, pyruvate and boiled tissue extract, increased the CO_2 output, interpreted this to mean that the vitamin is phosphorylated to cocarboxylase by the yeast enzymes in the presence of the extract. The results quoted above, however, indicate that the increased CO_2 production is due to the stimulation by the vitamin of the effect produced by the cocarboxylase present in the tissue extract. The fact that the pyrimidine components (which cannot be synthesized to cocarboxylase through lack of thiazole) also activate the cocarboxylase action makes it very unlikely that the effect of the vitamin itself is due to synthesis to cocarboxylase. That dry yeast does not phosphorylate vitamin B_1 to cocarboxylase to any extent is also indicated by the experiments of Kinnersley & Peters [1938]. The nature of the vitamin activation is not yet understood and is being further investigated.

Lipschitz *et al.* [1938] also reported that hexosediphosphate markedly increases the decarboxylation of pyruvic acid when boiled tissue extracts are present, an effect which is increased by addition of vitamin B_1; they supposed this action to be due to an influence on the enzymic synthesis of cocarboxylase from vitamin B_1. We find, however, that hexosediphosphate increases the CO_2 output in the complete absence of vitamin if both pure cocarboxylase and cozymase are present. The

effect increases with increasing concentrations of cocarboxylase[1] (Tables IV and V). Since accumulation of acetaldehyde is known to inhibit the enzymic decarboxylation of pyruvic acid the mechanism of action of hexosediphosphate is

Table IV. *Effect of hexosediphosphate on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of boiled extract from normal pigeon's liver, with and without addition of cocarboxylase.* (*Hexosediphosphate added, 7·8 mg. as Na salt*)

Sample	μl. CO_2 evolved in 2 hr.
Control	59
0·3 ml. boiled liver extract	235
0·3 ml. boiled liver extract + hexosediphosphate	293
0·3 ml. boiled liver extract + 0·5γ cocarboxylase	601
0·3 ml. boiled liver extract + 0·5γ cocarboxylase + HDP*	840

* HDP = Hexosediphosphate.

Table V. *Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase.* (*Hexosediphosphate added, 7·8 mg. as Na salt. Cozymase added, 0·2 mg.*)

	μl. CO_2 evolved in 2 hr.				
	γ cocarboxylase added				
Sample	0·5	1·0	2·0	3·0	0·5γ cocarb. + 5γ vit. B_1
Control	48	34	36	73	46
Cocarboxylase	192	361	667	790	427
Cocarb. + HDP*	162	334	657	783	394
Cocarb. + Coz.†	182	354	657	1036	435
Cocarb. + HDP* + Coz.†	200	533	1085	1400	701

* HDP = Hexosediphosphate. † Coz. = Cozymase.

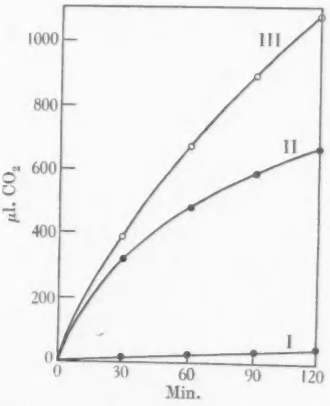


Fig. 2. Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in presence of cocarboxylase. Curve I, control. Curve II, 2γ cocarboxylase. Curve III, 2γ cocarboxylase + 7·8 mg. Na hexosediphosphate + 0·2 mg. cozymase.

clear. The yeast enzymes form triosephosphate which dismutates with acetaldehyde in the presence of cozymase to give ethyl alcohol and phosphoglyceric

[1] Essentially the same results have been obtained with brewer's yeast (Löwenbräu, Munich).

acid, so that acetaldehyde is removed and the decarboxylation can proceed at a higher rate. That this effect of hexosediphosphate should be unaffected by fluoride and inhibited by iodoacetic acid [cf. Lipschitz *et al.* 1938] is obvious and requires no further comment. When tissue extracts are added they supply the necessary cocarboxylase and cozymase. Fig. 2 shows that, in presence of hexosediphosphate and cozymase, the production of CO_2 drops less rapidly than it does with cocarboxylase alone, so that the effect of hexosediphosphate markedly increases with time.

2. *Activation by manganese*

Lohmann & Schuster [1937] found that either Mg^{++} or Mn^{++} are necessary for the decarboxylation of pyruvic acid by yeast. Mn^{++} was found by the above authors to be 5–10 times as effective as Mg^{++}, the largest amounts investigated being 10 and 100γ respectively. We find now that whereas 100γ Mg^{++} produce practically a maximum effect, the activation brought about by Mn^{++} continues to increase above 10γ up to approximately 100γ and may be very large (Fig. 3).

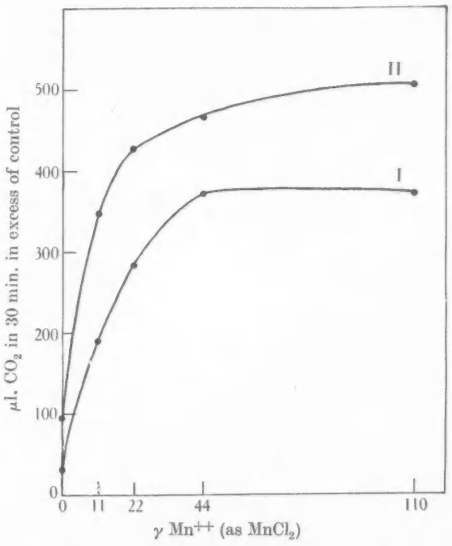


Fig. 3. Activation by Mn^{++} of the decarboxylation of pyruvic acid by alkaline washed yeast in presence of 1γ cocarboxylase, with and without vitamin B_1. Curve I, no vitamin. Curve II, 10γ vitamin B_1 hydrochloride.

This fact would only be of physiological significance if there is uneven distribution in the cell since 1 g. dry bottom yeast, Löwenbräu, Munich, contains only about 5·5γ Mn^{++} according to Ohlmeyer & Ochoa [1937], it is nevertheless interesting in regard to the mechanism of the reaction. Further, small amounts of Mn^{++} increase the activating effect of vitamin B_1. It should be noted that washing of our yeast by the method given above must remove most of the active ions, since if no Mg^{++} or Mn^{++} are added (in presence of cocarboxylase) no significant CO_2

production above the control (without cocarboxylase) takes place. This may account for the fact that if we add the Mg^{++} (100γ) mixed with the pyruvate, as Lohmann & Schuster did, we get very low CO_2 productions.

3. *Method of estimation of cocarboxylase and vitamin B_1*

The activation of the cocarboxylase action by vitamin B_1 makes it possible to determine the two compounds separately if present together. If an amount of vitamin B_1 which produces maximum (or nearly maximum) activation is added to the unknown solution, the cocarboxylase can be determined, since the CO_2 production will not be very much affected by the vitamin which was originally present. The addition of vitamin has also the effect of increasing very markedly the sensitivity of the method. Amounts of 0·01–0·02γ cocarboxylase can thus be estimated. For the cocarboxylase estimation 0·1 ml. vitamin B_1 solution in M/10 phosphate pH 6·2 (10γ vitamin B_1 hydrochloride) and 0·3 ml. unknown solution are added to the bottles, together with the other additions, and the CO_2 production is measured over a period of 30 min. The amount of cocarboxylase is found by reference to a curve obtained with pure cocarboxylase + 10γ vitamin B_1 (Fig. 4, curve II). In our experience, duplicates agree within the usual limits of

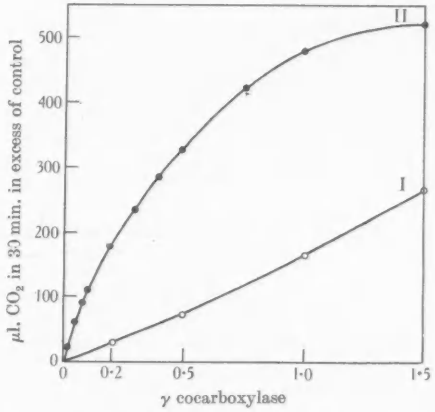


Fig. 4. Decarboxylation of pyruvic acid by alkaline washed yeast with increasing concentrations of cocarboxylase. Curve I, cocarboxylase alone. Curve II, cocarboxylase + 10γ vitamin B_1 hydrochloride.

error of manometric measurement. The assumption must be made that the action of boiled tissue extracts is due to the presence of cocarboxylase. This is at present reasonable as no other compound is yet known which has the same action as vitamin B_1 pyrophosphate.

For the estimation of vitamin B_1 the curve in Fig. 1 is used, working with a constant concentration of cocarboxylase. With an unknown extract, cocarboxylase is first determined, then extra cocarboxylase is added up to a total of 1·0γ in the bottles used. In this way CO_2 in excess of that produced with 1·0γ cocarboxylase alone represents CO_2 due to vitamin B_1 stimulation. The sensitivity of the vitamin estimation is much lower; amounts of vitamin under 0·05γ cannot be detected under our present experimental conditions.

With Mg^{++} (100γ) it is often found that there is little difference between the action of 0·1 and 0·2γ vitamin. In presence of a trace of Mn^{++}, however, the difference becomes distinct as the following experiment shows.

	μl. CO_2 in 30 min. (3γ Mn^{++} as $MnCl_2$. No Mg^{++})
0·5 cocarboxylase only	34·7 (triplicate)
0·5 cocarb. 0·1γ vitamin B_1	44·6 (triplicate)
0·5 cocarb. 0·2γ vitamin B_1	53·1 (duplicate)

This recent finding is being further explored.

Here the assumption that any stimulation by tissue extracts is due to vitamin B_1 cannot be made, as vitamin B_1 monophosphate would produce a similar effect. It is unlikely that free pyrimidines or other vitamin derivatives are present in sufficient concentrations to produce any effect. If, in what follows, we refer to free vitamin B_1 in tissue extracts, the possibility remains that the active substance may be partially or totally the monophosphate.

The main source of error in these methods is that the activity of the washed yeast may vary slightly from day to day or even from experiment to experiment, although washing and other experimental conditions have remained unaltered. It has been our custom therefore to check the cocarboxylase curve frequently and apply a correction if necessary. Some idea of the possible error may be got as follows: for a solution containing 0·25γ cocarboxylase and 1·3γ vitamin B_1 in 0·3 ml. an error of $\pm$0·02γ in estimating the cocarboxylase (an extreme case) might influence the vitamin estimation to the extent of $\pm$0·2γ, the vitamin value thus becoming 1·3$\pm$0·2γ. Our experience with yeast variations is curious. After initial rigid standardization, owing to the steady character of the results we relaxed our precautions of including known amounts of cocarboxylase and vitamin B_1 in each group of estimations; this saved much time. Latterly we have reinstated this precaution because at times unaccountable variations appear.

On the other hand, small variations in the amount of yeast in the bottles in a given experiment are without effect. A significantly lower CO_2 production (as compared with 1 ml.) has been found only with amounts of yeast suspension under 0·5 ml. as shown in Table VI.

Table VI. *Decarboxylation of pyruvic acid by alkaline washed yeast using various amounts of yeast suspension*

Sample	Time min.	μl. CO_2 1·0 ml. washed yeast	0·5 ml. washed yeast	0·25 ml. washed yeast
0·5γ cocarboxylase	30	72	73	56
0·5γ cocarb. + 10γ vitamin B_1	30	323	293	247
0·5γ cocarboxylase	60	111	115	91
0·5γ cocarb. + 10γ vitamin B_1	60	468	449	383

Recoveries of cocarboxylase and vitamin B_1 added together to various tissues are shown in Table VII. There is a tendency for rather high recoveries with small amounts of cocarboxylase in presence of large amounts of vitamin. Although this is not yet understood, the results show that no significant destruction of either compound takes place during the operations involved in the preparation of the boiled extracts. It must be realized that only 1/9[1] of the amounts added per g. tissue are actually estimated, i.e. in line 5 (Table VII) about 0·14γ cocarboxylase.

It may be added that the method suggested by Lipschitz *et al.* [1938] cannot lead to the desired separate determination of cocarboxylase and vitamin B_1, because it involves for the former the use of iodoacetic acid, which we have shown not to inhibit the vitamin activation, and for vitamin B_1 the use of

hexosediphosphate which can increase CO_2 production in the complete absence of free vitamin.

Table VII. *Recovery of cocarboxylase and vitamin B_1 added together to minced normal pigeon's tissues before preparing boiled extracts therefrom (cocarboxylase and vitamin B_1 are expressed in γ per g. fresh tissue)*

	Cocarboxylase, γ				Vitamin B_1, γ			
Tissue	Added	Found	Recovered	%	Added	Found	Recovered	%
Muscle	0·00	4·14	—	—	0·00	2·43	—	—
	0·75	5·20	1·06	141	18·70	19·50	17·07	91
	3·28	7·20	3·06	93	1·64	4·15	1·72	105
Liver	0·00	4·85	—	—	0·00	0·90	—	—
	1·30	7·02	2·17	167	32·50	32·40	31·50	97
	5·00	9·55	4·70	94	2·51	—	—	—
	0·00	4·38	—	—	0·00	0·99	—	—
	5·00	8·52	4·14	83	5·00	5·68	4·69	94
	2·50	7·30	2·92	117	25·00	20·70	19·71	79
	0·00	4·23	—	—	0·00	—	—	—
	4·00	8·18	3·95	99	8·00	—	—	—

4. *Cocarboxylase and vitamin B_1 contents of animal tissues*

In early experiments Simola [1932] found a decrease in cocarboxylase (about −60 %) in the brain and liver of rats upon synthetic diets as compared with those on normal diets; in the absence of vitamin B the amounts were further reduced; the cozymase content did not vary. Westenbrink [1934] and Leong [1937] have reported estimates of vitamin B_1, by weight and bradycardia tests respectively, in rat tissues; presumably such determinations should include cocarboxylase. Leong's principal values were for rats upon ordinary diet: liver 3·75, heart 3·5, brain 3·5, muscle 1·0γ vitamin B_1 per g. tissue, upon the assumption that 1 international unit = 2·5γ vitamin B_1.

Preparation of boiled extracts. Pieces of pigeon and rat tissues were weighed in weighing bottles, minced or chopped and ground in a mortar with 2 vol. distilled water; the suspension was then heated for 3–5 min. in a boiling water bath with stirring, cooled and centrifuged. The brain, as well as the liver, was sometimes minced on an ice-cold plate, but the results so obtained did not differ from those reached by mincing at room temperature. In the case of one animal the brain was removed under amytal anaesthesia after freezing *in situ* with liquid air [Kerr, 1935]; again there was no significant difference. We think therefore that cocarboxylase remains comparatively stable at room temperature, though we know that slight disappearance occurs on incubation at 38°.

Estimations have been performed upon 0·3 ml. samples of extracts from brain, muscle, liver and heart in animals taken as follows.

Pigeons.

I. Normal.

II. Vitamin B_1-deficient (with symptoms).

III. Rice-fed for 25 days (no symptoms).

IV. Vitamin B_1-deficient (with symptoms) and injected with vitamin B_1 (1 mg. per 100 g. animal) 25 min. before death.

V. Vitamin B_1-deficient (with symptoms) and dosed by mouth with vitamin B_1 (100γ *per diem* per animal) for 3 days before death.

VI. Rice-fed for 26 days (no symptoms) but treated as animals of group V for 3 days before death.

Rats.

I. Normal.

II. Vitamin B_1-deficient (with symptoms).

III. Same as II but vitamin B_1 injected (1 mg. per 100 g. animal) 45 min. before death.

IV. Normal or avitaminous but vitamin B_1 given by mouth (1 mg. per 100 g. animal) 1 to 2 hr. before death.

All animals were killed by decapitation and bled before taking tissue samples.

Birds which have been upon a polished rice diet for 25 days and over and which are receiving daily doses of vitamin B_1, are perfectly well in appearance but they do not regain weight significantly. This is due to lack of other nutritional factors [Carter & O'Brien, 1935; 1936]. They form a good control for the birds with symptoms because of their generally normal behaviour at a much diminished body weight (about 70% of the normal). 100γ vitamin B_1 *per diem* is a comparatively large dose as 10γ would already produce curative effects.

Results. The average values for pigeons are given in Table VIII and individual values of rats in Tables IX and X. The values for pigeon's brain and liver are shown graphically in Figs. 5 and 6. Individual results for pigeons are given

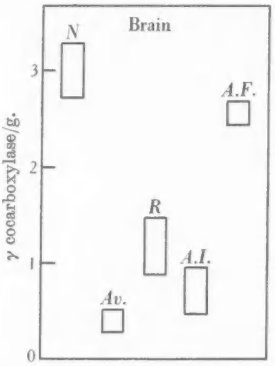


Fig. 5.

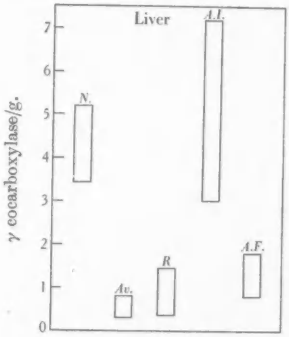


Fig. 6.

Fig. 5. Cocarboxylase in pigeon's brain. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days. Ordinates: mean $\pm 2\epsilon$.

Fig. 6. Cocarboxylase in pigeon's liver. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days.

in the appendix. $\pm 2\epsilon$ (the standard error of the mean) represents $\sigma/\sqrt{n}$ for 6 estimations and over, but $\sigma/\sqrt{(n-1)}$ for fewer estimations than 6. Values are calculated upon the assumption that the fresh tissues contain 70% H_2O.

A more accurate value would be for brain 80%, liver 72%, muscle 74%, based upon the figures of Roche [1925]: brain, normal 80·7, avitaminous 79·9, inanition 80·2. Liver, normal 71·3, avit. 71·6, inan. 72·6. Muscle, normal 74·8, avit. 73·7, inan. 75·4. A few estimations by us confirm these values. The extreme variations for Roche's tissues were small. For truer values our brain results can be raised by 5% approx. and the muscle values by 1·5%.

Though we cannot claim close accuracy for our present method and think it possible that further work may bring to light other sources of error than those referred to above (p. 1503) we consider that our determinations are sufficiently good to demonstrate the points at issue. Even if 50 % of the cocarboxylase estimations (cf. Table VIII) were in error to the extent of +50 % this would mean that the values in Table VIII would be higher by 25 %, but the conclusions below would still stand. As to the vitamin results we can obtain no evidence for the presence of free vitamin B_1 (or its monophosphate) in normal or avitaminous brain, whereas it can be detected after administration. This is therefore a clear difference, but in view of the lower sensitivity of the vitamin method (see p. 1507) it can only be stated definitely that there is less than 0·45γ per g. present.

In the case of the results with brain, and occasionally with liver, showing no vitamin B_1, it has sometimes happened that the tissue extract gave slightly less CO_2 than the control with 1γ cocarboxylase. This has been within the limits of experimental error, so that there is no reason to suspect the presence of inhibitory substances.

In the case of the cocarboxylase values for liver in group IV (Table VIII), it will be seen that they are upon the average over 10 times larger than the corresponding ones of group II. This cannot be due to error introduced by the presence of large amounts of free vitamin, since this (as judged from Table VII) could not raise the results more than 1·7 times.

The outstanding points to note in these results are:

(1) There is much less vitamin B_1 than cocarboxylase in the liver and brain.

(2) The cocarboxylase is reduced in all tissues in both the avitaminous and the rice-fed pigeon, as well as in the avitaminous rat.

Table VIII. *Cocarboxylase and vitamin B_1 in pigeon's tissues (values expressed in γ per g. fresh tissue)*

			Cocarboxylase			Vitamin B_1		
Group	Condition of birds	Tissue	No. of obs.	Mean	Standard error $\pm 2\epsilon$	No. of obs.	Mean	Standard error $\pm 2\epsilon$
I	Normal	Brain	12	3·00	±0·28	7	0·00	±0
		Muscle	9	3·81	±0·42	7	1·60	±0·50
		Liver	9	4·33	±0·88	7	0·32	±0·30
		Heart	8	4·36	±0·57	5	1·54	±0·75
II	Avitaminous (symptoms)	Brain	9	0·40	±0·12	6	0·00	±0
		Muscle	6	1·04	±0·56	5	0·96	±0·71
		Liver	9	0·48	±0·30	7	0·20	±0·20
		Heart	6	0·55	±0·47	5	0·45	±0·27
III	Rice-fed (no symptoms)	Brain	8	1·18	±0·31	—	—	—
		Muscle	2	0·81	—	—	—	—
		Liver	3	0·73	—	—	—	—
		Heart	4	0·91	—	—	—	—
IV	Avitaminous, vitamin B_1 injected 25 min. before death	Brain	4	0·72	±0·24	4	0·55	±0·27
		Muscle	4	2·20	±0·37	4	3·04	±1·90
		Liver	4	**5·10**	±2·10	4	**14·20**	±10·20
		Heart	4	2·05	±0·52	4	4·05	±1·50
V	Avitaminous, dosed 3 days with vitamin B_1	Brain	6	2·55	±0·12	—	—	—
		Muscle	6	1·55	±0·20	—	—	—
		Liver	6	1·28	±0·51	—	—	—
		Heart	6	2·46	±0·49	—	—	—
VI	Rice-fed (no symptoms), dosed 3 days with vitamin B_1	Brain	4	2·82	—	—	—	—
		Heart	4	2·45	—	—	—	—

(3) There is a large increase in both cocarboxylase and vitamin B_1 in the livers of animals shortly after administration of vitamin.

(4) There is a much larger relative increase in the cocarboxylase in brain (and heart) than in the other tissues in pigeons dosed with smaller amounts of vitamin over a period of 3 days. In fact practically normal values are found.

(5) The brain cocarboxylase of pigeons not showing symptoms is higher than that of those which do show symptoms.

Table IX. *Cocarboxylase and vitamin B_1 in rat's tissues. (Individual values. Expressed in γ per g. fresh tissue)*

		Brain		Muscle		Liver		Heart	
Group and condition		Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
I	Normal	2·93	0·00	0·38	0·90	5·40	0·60	4·15	—
	,,	2·91	0·20	0·32	0·50	4·85	0·60	3·50	0·60
II	Avitaminous	0·68	—	0·10	—	0·53	—	0·52	—
	,,	0·53	—	0·11	—	0·36	—	0·31	—
III	Avitaminous, vitamin B_1 injected	0·72	—	0·16	3·80	6·30	24·30	0·74	—
		0·54	0·50	0·13	—	2·91	84·60	0·67	—
		0·73	—	0·20	1·10	8·62	7·40	0·77	—

Table X. *Cocarboxylase and vitamin B_1 in the liver of rats after oral administration of vitamin B_1 (Group IV); individual values*

Condition	Animal killed after (hr.)	Cocarboxylase γ per g.	Vitamin B_1 γ per g.
Normal	1	6·50	3·40
Avitaminous	2	6·30	8·10
Avitaminous	2	8·40	8·30

Discussion

We interpret the above facts to mean that cocarboxylase is a very significant form of vitamin B_1 in animal tissues. In some, such as brain and liver, it appears to be predominant; in skeletal and heart muscle there is relatively more vitamin B_1 present, which sometimes approaches the amount of cocarboxylase, hence we cannot say dogmatically that vitamin B_1 itself may not also have some normal function in life. In rat muscle the amount of cocarboxylase is surprisingly low, but there is not much vitamin B_1 either; this agrees with the findings of Westenbrink [1934] and Leong [1937]. As will be noticed by comparing groups II, IV and V of Table VIII, vitamin B_1, if administered, is taken up by the liver and rapidly synthesized to cocarboxylase; with small doses over a period of several days the amount present in the liver again decreases and a preferential increase takes place in the brain and heart, i.e. in the two tissues which are specially affected by vitamin B_1 deficiency. There must therefore be a special affinity for heart and brain tissues reflected in their biological needs. It is evident that even considerable reduction (down to 40 % of the normal) in the cocarboxylase content of the brain can occur before the function of its cells is impaired as shown by the appearance of symptoms. So far as this implies that these symptoms are associated with a low brain cocarboxylase, the evidence reinforces previous views from this laboratory as to the dependence of normal function upon biochemical state. But it is a new point that the liver may co-operate.

What we now want to know is whether in normal functioning there is any local synthesis by brain tissue or whether all the cocarboxylase is transported as such in the blood. The fact that in some birds there is a massive synthesis of

cocarboxylase in the liver before the symptoms clear up is not decisive, because either any local synthesis might be slow, or the cells might take an appreciable time to re-establish sufficient normality.

The finding of Sinclair [1938] that the vitamin B_1 in the blood is in a combined form, though admitting of other interpretations, is consistent with its conveyance as the pyrophosphoric ester; nevertheless there is difficulty in understanding how such an ester could permeate brain and other tissue cells, a difficulty not lessened by the failure to confirm the equal catatorulin activity of cocarboxylase.

We may ask whether the intestine phosphorylates vitamin B_1 *in vivo* in a similar fashion to that suggested for glucose and fat by Verzár & McDougall [1936] or for lactoflavin by the experiments of Rudy [1936]. This needs some direct test, but the experiments of Table X indicate that much vitamin B_1 as such can reach the liver; they are therefore indirect evidence against obligate phosphorylation in the intestine. This conclusion is supported by the failure to obtain *in vitro* phosphorylation of the vitamin with intestinal mucosa, as already referred to in the introduction (unpublished experiments). There seems to be little doubt that the proof that the liver participates in the metabolism of this vitamin is important and suggestive from the clinical standpoint. Its full understanding must wait for more accurate knowledge of the metabolism of pyruvic acid.

Summary

1. Vitamin B_1 (as also does its monophosphoric ester) stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of cocarboxylase. This action is due to the pyrimidine half of the molecule containing the NH_2 group. It is not due to removal of acetaldehyde by oxidation or dismutation. The nature of this activation is not yet understood and is being further investigated.

2. Hexosediphosphate increases the rate of decarboxylation in presence of cozymase and pure cocarboxylase in the total absence of vitamin B_1. The mechanism of this effect is discussed.

3. Mn^{++} greatly stimulates the carboxylase system if present in sufficient concentrations.

4. The activation of cocarboxylase action by vitamin B_1 has been made use of in a method for the separate estimation of cocarboxylase and free vitamin in the same solution or tissue extract.

5. There is much less vitamin B_1 than cocarboxylase present in boiled extracts from rat and pigeon brain and liver; both occur in those from muscle and heart tissues.

6. The cocarboxylase content of tissues is much reduced in the B_1-avitaminous condition and specifically so in the brain tissue, being soon increased in the latter (and also in the heart) after a short period of administration of the vitamin.

7. Administration of vitamin B_1 to animals leads to an immediate accumulation of both vitamin B_1 and its pyrophosphoric ester in the liver. For the first time this brings the liver into prominence in the metabolism of vitamin B_1.

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Appendix

Table XI. *Cocarboxylase and vitamin B_1 in tissues from normal pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
3·60	—	4·90	—	2·70	—	4·60	—
3·40	0	3·40	0·90	4·05	0·50	5·22	0·80
2·44	0	—	—	—	—	—	—
2·61	0	—	—	—	—	—	—
2·60	0	2·97	2·25	2·80	0·54	2·90	2·70
2·60	—	4·50	1·60	5·00	—	5·20	1·30
2·96	—	3·70	1·60	3·95	0·00	4·05	—
2·96	—	3·33	0·80	4·40	0·30	5·10	—
3·87	—	2·87	1·80	7·20	0·00	3·38	1·80
3·42	0	4·95	—	4·05	0·00	4·46	1·08
2·76	0	—	—	—	—	—	—
2·70	0	—	—	—	—	—	—
—	—	4·14	2·43	4·85	0·90	—	—

Table XII. *Cocarboxylase and vitamin B_1 in tissues from avitaminous pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
0·54	—	2·35	—	1·00	—	1·70	—
0·45	0	0·90	1·20	0·31	0·60	0·36	0·54
0·35	0	1·08	2·10	0·18	0·50	0·45	0·72
0·18	—	0·63	0·40	0·22	0·00	0·18	0·00
0·27	0	0·65	0·50	0·15	0·00	0·21	0·54
0·18	0	0·63	0·60	0·34	0·00	0·40	0·45
0·72	0	—	—	0·23	0·30	—	—
0·40	0	—	—	1·50	0·00	—	—
—	—	—	—	0·40	—	—	—
0·54	—	—	—	—	—	—	—

Table XIII. *Cocarboxylase in tissues from rice-fed pigeons (no symptoms) (γ per g.)*

Brain	Muscle	Liver	Heart
0·99	—	0·45	—
2·20	0·54	0·31	0·99
0·93	1·08	1·44	0·63
0·99	—	—	0·68
0·90	—	—	1·35
1·22	—	—	—
0·95	—	—	—
1·28	—	—	—

Table XIV. *Cocarboxylase and vitamin B_1 in tissues from avitaminous pigeons a short time after injection of vitamin B_1 (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B
0·84	0·40	2·50	5·40	5·75	19·00	2·50	3·60
0·72	0·40	2·07	2·70	2·34	9·50	2·07	4·50
0·42*	0·50	2·40	2·70	5·93	24·00	1·44	5·60
0·90*	0·90	1·80	1·35	6·30	4·50	2·20	2·50
0·81†	0·00	1·71	0·90	2·34	2·70	2·34	2·70
0·54‡	—	0·90	—	1·35	—	0·54	—

* Not recovered at the end of 25 min.
† Bird killed after 1 hr. Not included in average.
‡ Only 25γ vitamin B_1 HCl injected. Killed 25 min. after recovery. Not included in average.

Table XV. *Cocarboxylase in tissues from rice-fed pigeons (19–27 days, with symptoms) dosed by mouth for 3 days with 100γ vitamin B_1 per day*

Date	No. days on rice	Initial wt. g.	Final wt. g.	Wt. after B_1 dosing	γ cocarboxylase per g.			
					Brain	Muscle	Liver	Heart
28 April	27	340	202	203	2·70	1·86	0·81	1·90
	27	402	262	258	2·75	1·26	2·11	1·80
	27	473	316	328	2·43	1·35	0·86	2·70
20 May	19	340	231	232	2·54	1·41	0·92	3·20
	19	371	244	255	2·43	1·35	0·90	3·02
	27	280	194	212	2·43	2·03	2·07	2·12
Average		368	241	248				
Av. % fall		—	65	67				
Av. % increase		—	—	2				

Table XVI. *Rice-fed birds dosed with 100γ vitamin B_1 on successive days*

No. days on rice	Initial wt. g.	Final wt. g.	Wt. after 3 days dosing	γ cocarboxylase per g.	
				Brain	Heart
26	357	252	274	3·40	2·30
26	388	270	299	2·75	1·90
26	395	278	293	2·25	2·90
26	316	210	221	2·90	2·70
Average	364	252	272	2·82	2·45

Av. % fall 69. Av. % rise 6.

Note added 23 August 1938. Similar results to ours for cocarboxylase in tissues have been reached simultaneously by Westenbrink & Goudsmit (*Nature*, 1938, **142**, 151).

CXCIX. IMPROVEMENTS IN THE USE OF THE FORMALDEHYDE AZO REACTION FOR VITAMIN B_1

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This paper embodies further observations made upon and in relation to the formaldehyde azo reaction for vitamin B_1 [Kinnersley & Peters, 1934] and its possible application to semi-quantitative estimation of vitamin B_1. The development of the more sensitive thiochrome method of estimation [Jansen, 1936; Karrer & Kubli, 1937; Pyke, 1938] during the progress of this work, may ultimately make the method here described obsolete. Nevertheless, we think that some independent check may prove useful, and further, that the method may prove of value, when enough vitamin B_1 is available, owing to the stability of the standards, and to the fact that a dark room and a source of ultra-violet light are not required. Unfortunately, neither method is successful with phosphoric esters of vitamin B_1; these do not give the formaldehyde azo test (as stated by Lohmann & Schuster [1937]); they form a blue fluorescent compound with alkaline ferricyanide, but this does not pass into a butyl alcohol layer, which is a basis of separation from interfering fluorescent substances [Kinnersley & Peters, 1937]. We have found that phosphatase can be used in the pre-treatment of the extracts to liberate the vitamin B_1. In our previous communication we stated that the condition of alkalinity chosen and the presence of formaldehyde largely reduced interference by other substances; a particularly troublesome impurity giving a pseudo-vitamin B_1 colour reaction could be distinguished by its relative instability in acid solution. We have realized for some time that butyl alcohol would extract the bulk of the pink substance formed in the reaction. By an error the colour was compared in our previous paper [1934] with phenol red in phosphate buffer *p*H 5; it should have been "approximately *p*H 7·3". A considerable improvement is again made by shaking the butyl alcohol with dilute acid, when the pink substance passes back into the acid aqueous phase; the whole test now compares favourably as regards specificity with other methods and can be readily applied to vitamin B_1 concentrates from the International Standard Clay (1936) and also the cruder "charcoal" concentrates. We have found certain conditions which interfere with this and other azo tests. The work falls into two parts:

I. The method and some conditions modifying it;

II. Application to crude vitamin B_1 extracts.

I. *The method and some conditions modifying it*

(*a*) *The original formaldehyde azo test has been modified as follows.*

To 0·1–0·3 ml. of a solution containing 10–20γ vitamin B_1 (acidity best about *p*H 6·0) and 30 % ethyl alcohol, is added after one minute a mixture of 1·25 ml. of the special reagent and 0·5 ml. diazotized sulphanilic acid. The alcohol now included has proved essential for full development of the pink colour [cf. Koessler & Hanke, 1919]. After standing for 2 hr. or more, the pink-coloured

solution is extracted twice successively with 2 ml. of butyl alcohol. The combined butyl alcohol extracts are then extracted with 2 ml. $N/200$ HCl and with 1 ml. twice (this should make the *p*H of the aqueous phase about 6·5). If not acid after shaking, more acid (trace of $N/10$) must be added; the pink HCl phase is now removed, and an equal volume of ethyl alcohol is added. The resulting coloured solution is compared with standards of vitamin B_1 similarly treated. They should be kept out of strong light except during tests. We have tried the colorimeter (visual and photoelectric), but are convinced that equal and even better accuracy in these dilute solutions is obtained by a naked eye comparison of similar small bore tubes. Our estimate of the accuracy of the method is $\pm 5\%$ for amounts of aneurin HCl of approximately 20γ. Standards of 40γ and over can be prepared.

(*b*) *Conditions modifying the test*

During our experience with this test some facts have been discovered which are briefly recorded. Reducing agents such as cysteine or traces of sodium hydrosulphite, markedly delay the appearance of colour. Traces of some metals interfere both with azo reactions of the Pauly type for histamine and histidine, and also with the reaction for vitamin B_1.[1] Other systems have not been investigated. We do not know of any previous publication showing this effect upon azo reactions.

Histidine. Estimating 20 μg. by the usual Pauly reaction according to the technique of Koessler & Hanke [1919], no interference was produced by U, Mn, Nb, Rb, Ta, Be, Pb, La, Th, Thallium, V, Cs, Cr, Fe in amounts of 20γ, or by Al 10γ, or In 25γ; interference was produced by Ni 5γ, Co 10γ, Hg 20γ, Mn slightly, Cd 100γ, Sn 9γ, Ce 20γ slight, Zn 10γ, Cu 3γ. The metals interfere mostly by reducing pinkness in the solution.

Vitamin B_1 formaldehyde azo reaction. Remarkably little interference was produced by most metals tried. Exceptions were found in Cu, Hg, Ag. With 10γ vitamin B_1 Cl, HCl, 4γ Hg were inhibitory and 10γ Ag slightly so. The effect of Cu was of the greatest interest. A delayed reaction developed with minute traces of this, an amount equivalent to one atomic weight per mol. producing practically maximal effect. The action depends upon the presence of formaldehyde.

The question arises whether the presence of a metal could explain the delayed azo reaction previously reported by Kinnersley *et al.* [1935] during the formation of quinochromes by oxidation with manganese oxides. If Mn compounds could produce the effect this would be reasonable; but they do not do so, nor is it possible to modify the reaction for vitamin B_1 by addition of the solution in which the vitamin is oxidising. Hence the original interpretation that the delayed azo reaction indicates a change in the vitamin molecule is still tenable.

II. *Application of reaction to crude vitamin B_1 extracts*

We have made a preliminary attempt to apply this to certain food materials, as well as to vitamin B concentrates, using for biological tests the birds required in the course of another research. After considerable trial, 50% acid ethyl alcohol has been employed to extract the crude, ground foodstuff. Both vitamin B_1 and its pyrophosphoric ester are very soluble in 50% ethyl alcohol and stable in faintly acid solution; 50% acid alcohol also removes vitamin B_1 from adsorbents such as charcoal. From what is now known of the chemistry of these substances it seems to us to be inconceivable that this extraction is not adequate; tests with 75% alcohol have not shown any larger recovery of the vitamin. In our experience it is essential to concentrate the vitamin and to remove it from certain interfering substances. For this purpose we cannot improve upon sodium phosphotungstate, which has been long used in this laboratory for the preparation of

[1] Some of these reactions were shown to the Biochemical Society in May 1936.

vitamin B_1 and which is known to precipitate it in very low concentration. The complete method is as follows.

A convenient amount (100 g. in the case of oatmeal) of the finely ground foodstuff is treated with 1000 ml. of boiling water; 2 ml. conc. HCl are added to bring it to *p*H 3·5, and the whole is heated for a few minutes to boiling. The rather thick mixture is placed on the boiling water bath for an hour or more and enough ethyl alcohol is then added gradually to make it up to 50 % by volume. The whole is filtered after cooling. Some materials require more solvents than others. The extract, after cooling overnight, and being freed from precipitate by Büchner filtration, is evaporated to 150 ml.; the water bath suffices for this as this vitamin is relatively stable in dilute acid solution. After further cooling and removal of insoluble matter by the centrifuge, Na phosphotungstate, 10 % at *p*H 6, is added to slight excess and the mixture is acidified to *p*H 1·0 with 20 % H_2SO_4. The phosphotungstate precipitate is allowed to stand 12 hr., collected in the centrifuge tube, and ground with baryta three times, according to our usual technique [Kinnersley *et al.* 1935]. After removal of baryta by H_2SO_4, the extract is concentrated if necessary at *p*H 3, care being taken to see that greater acidity does not develop during concentration; any free H_2SO_4 should be removed by $BaCl_2$ during this process. A convenient volume not exceeding 0·3 ml. is taken for the test.

Table I. *Comparison of vitamin B_1 in food materials by colour and biological tests.* (*γ per g. or ml. test material undried; no. of birds used in parentheses*)

Food material	Moisture content %	γ vitamin estimated by: Colour test	γ vitamin estimated by: Bird test	Bird test: g. food given	Bird test: γ vitamin found	±2ε (γ)
1. Yeast concentrate 41	—	120·0	145·00 (153)			
2. Wheat germ	7·0	20·0	13·50 (11)			
3. Pea	11·65	3·5	2·94 (81)	6	1·95 (11)	
				3	3·27 (45)	2·72–3·97
				5	2·80 (25)	2·22–3·55
4. Breakfast oats	10·0	3·0	2·54 (48)	6	2·23 (16)	
				5	2·75 (12)	
				3	2·67 (20)	2·39–2·98
5. Haricot bean	12·7	2·2	2·15 (5)			
6. Oatmeal	10·3	2·0	1·87 (41)	6	2·00 (20)	
				5	1·75 (21)	
7. Maize	9·7	2·0	1·54 (55)	6	1·41 (26)	1·09–1·82
				10	1·12 (6)	
				5	1·80 (23)	
8. Lentil	12·7	1·75	1·60 (12)			
9. Barley	12·0	1·2	0·71 (11)			

NOTES:

1. Yeast concentrate 41 was a 50 % alcohol concentrate from charcoal used in routine work in the Laboratory.
2. The average day dose for the birds is calculated by the logarithmic method [Kinnersley & Peters, 1936].
3. The day dose is taken as equivalent to 2·5γ.
4. The individual tests are not here included, in order to economize space. They can be made available by writing to this laboratory.
5. In a few cases, the values of ±2ε are quoted as a guide. For barley there was wide variation in the 11 tests of which the average is given.
6. Values for assays of vitamin B_1 quoted in the review by Fixsen & Roscoe [1938] are wheat germ 10–55γ, pea 1·0–3·0γ, haricot bean 1·3–2·5γ, oatmeal 8γ, breakfast oats 3·5γ, whole maize 0·75–1·5γ, lentil 1·0–5·2γ, all per g.

Using the pigeons available in the laboratory in the course of another research, we have obtained the following preliminary results. So far as we know the curative and protective test is valid for these cruder concentrates, though Kinnersley & Peters [1936] showed that it would not apply to the pure vitamin. The figures given in columns 5 and 6 of Table I give reasonably satisfactory agreement between the larger and smaller doses except in the case of the 6 g. dose for the pea, but here it will be noted that the number of tests is fewer. Inspection of Table I shows that parallel results are obtained with the azo method and biological tests; the order of arrangement in the table is based on the colour test and it agrees with that obtained by the bird test. The results of the bird tests have been calculated upon the assumption that one day dose equals $2{\cdot}5\gamma$ vitamin B_1; in 1936, we concluded that one day dose must be equivalent to $2{\cdot}0\gamma$ *per diem* as the result of tests of the catatorulin type and also of colour reactions. Since then, however, we have found that our extract of the acid clay may have deteriorated and that a better value is $2{\cdot}5\gamma$ (cf. Sampson & Keresztesy [1937], also Pyke [1938]). Though there are some marked deviations from previous estimates of similar foodstuffs in the literature (see note 6 to Table I), our results as a whole are of the same order.

Phosphoric esters of vitamin B_1 (cocarboxylase etc.). We have attempted to decide how far the presence of phosphoric esters of vitamin B_1 interfere in the case of (*a*) yeast extracts and (*b*) foodstuffs. The most suitable method for breaking up the phospho-compounds is enzymic. After considerable trial, and with the final advice of Dr Kay to whom we are grateful, we adopted takaphosphatase (as present in takadiastase 0·5 %), acting at approximately *p*H 4. The result of one experiment is given in Table II.

Table II. *Effect of takadiastase on cocarboxylase (crude)*

Exp. 1. Solutions A, B, C were made up as stated below and incubated at 38° for one hour, after which the vitamin B_1 and phospho-esters were determined by a thiochrome method upon an aliquot. The results in the Table are expressed as equivalents of vitamin B_1.

Solution	A	B	C
Takadiastase	100 mg. in 10 ml.	Nil	100 mg. in 5 ml.
Cocarboxylase	20 ml.	10 ml.	Nil
Phosphate buffer *p*H 4	50 ml.	25 ml.	12·5 ml.
Water	Nil	5 ml.	Nil
Content in esterified or free vitamin B_1			
Before incubation:			
Vitamin B_1: Esterified	600	600	Nil
Free	100	100	Nil
After incubation:			
Vitamin B_1: Esterified	50*	500	Nil
Free	500*	100	Nil
% "free" vitamin liberated by phosphatase	91	15	—

* These values were checked by azo colour tests which were in agreement. The crude cocarboxylase solution contained preformed vitamin B_1.

Both by azo test and fluorescence it was found that approximately 90 % of the combined vitamin B_1 had been converted into the free form by this treatment.

Application of this treatment to yeast extracts showed that in the initial extracts combined as well as free vitamin B_1 might be present, but there was no reason to think that this applied to the foods. Hence this possible objection to the use of both the azo test and the modification of Jansen's thiochrome methods in these foodstuffs appears in practice to be without foundation.

Though vitamin B_1 added can be recovered quantitatively, it is conceivable that some of the vitamin B_1 present in various forms in the foodstuffs may escape extraction with dilute acid or precipitation by phosphotungstate. But any such amounts are likely to be small in view of the known properties of the vitamin and its compounds. In any case, a method of this nature is valuable chiefly to establish a minimum. In view of the fair parallelism seen in Table I, we think that the method merits further exploration.

Summary

1. The formaldehyde azo test for vitamin B_1 has been improved and extended to include a preliminary investigation of the vitamin content of foodstuffs, in which colour tests have been compared with biological tests. Parallel results have been obtained.

2. Certain metals in small amounts interfere with this test, and also with reactions of the Pauly type.

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CC. THE IDENTITY AND MECHANISM OF ACTION OF THE GLYCOTROPIC (ANTI-INSULIN) SUBSTANCE OF THE ANTERIOR PITUITARY GLAND

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THE experiments of Cope & Marks [1934] were the first to demonstrate that the injection of a crude anterior pituitary extract into a normal rabbit can induce complete insensitivity to the hypoglycaemic action of insulin administered many hours after the last injection of pituitary extract. In the experiments of these authors the blood sugar level of the treated rabbit was seldom significantly affected by the injections of the anterior pituitary preparation alone, and the "anti-insulin" activity of the extracts was therefore not due to a "diabetogenic" action. For the purpose of convenience the name "glycotropic factor" was suggested [Young, 1936, 1] for the anterior pituitary substance which induces insulin insensitivity without itself causing a rise of blood sugar level. Investigation showed [Young, 1936, 1] that the glycotropic factor was present in preparations of prolactin obtained by the method of Riddle *et al.* [1933], a fact which appeared to be of significance with respect to the action of anterior pituitary preparations on milk secretion [Young, 1936, 1, 2]. Subsequent observations [Young, 1937, 1; 1938] showed that prolactin and the glycotropic substance are not identical. The evidence presented in support of this differentiation, although conclusive, has previously been described only briefly, and it is one purpose of the present communication to supplement the description of the earlier work and to present further evidence that prolactin and the glycotropic factor are not identical. The results of experiments on the influence of the administration of anterior lobe extracts on the hyperglycaemic action of injected adrenaline and on sugar tolerance are also recorded in the present paper, and the significance of the results obtained is discussed with respect to the mechanism of action of the glycotropic substance.

EXPERIMENTAL

Animals

The animals used in the majority of the experiments in the present investigation have been unanaesthetized, healthy, young adult rabbits, male or female, weighing about 1·5 kg., from an inbred pure strain of Dutch rabbits bred at the farm laboratories of the National Institute for Medical Research. In our experience animals of this strain are particularly constant in their response to glycotropic pituitary extracts.

In the investigations on the influence of the subcutaneous injection or intravenous infusion of adrenaline it was convenient to use larger rabbits, so pure strain Chinchilla rabbits, and occasionally rabbits weighing about 3 kg. purchased from a dealer, were used for these experiments.

[1] Work begun during the tenure of a Beit Memorial Fellowship.

All the rabbits received an ample diet of cabbage, bran, oats and hay. For experiments on the glycotropic factor it is essential to use rabbits which are in excellent condition and are eating well, in order to obtain consistent results.

Biological assays

Thyrotropic activity was assayed by the method of Rowlands & Parkes [1934]. The thyrotropic activity considered in the present investigations is therefore that causing an increase in size of the thyroid gland of the immature guinea-pig. It is not certain that the substance active in this respect is identical with that stimulating secretory activity of the thyroid gland.

Prolactin was assayed by the pigeon crop-gland method according to the technique described by Rowlands [1937]. The name prolactin is used to describe the substance active in causing increase in weight of the pigeon crop-gland, without reference to the question whether or not all preparations of this substance are truly lactogenic [cf. Folley & Young, 1938].

Method of expressing the results of biological assays

The question of establishing International standard preparations of prolactin and of the thyrotropic hormone, and of establishing International units of activity in terms of these preparations, is at present under discussion. Any method of expressing the physiological activity of pituitary substances in terms of units, in the present paper, must therefore be regarded as of temporary value only. In connexion with the question of establishing an International standard preparation of prolactin, Dr O. Riddle and Dr R. W. Bates have kindly made available a preparation of prolactin standardized by them. An assay curve for this prolactin, which contains 10 Riddle units/mg., has been constructed by the methods used in this laboratory. This curve has allowed us to express the results of our pigeon crop-gland assays in terms of Riddle units, and in the present paper this has been done.

Rowlands & Parkes [1934] defined a unit of thyrotropic activity as that amount of thyrotropic substance which, when injected daily for 5 days, would induce the thyroid glands of a 200 g. guinea-pig to double in weight. This unit has been adopted in the present paper, and will be described as the Rowlands-Parkes unit of thyrotropic activity.

Blood sugar estimations

Blood sugar was determined on 0·1 ml. of blood by the method of Hagedorn & Jensen. The figures in the present paper are therefore higher than those for "true" blood sugar.

In our experience the blood sugar level of a pituitary-treated rabbit is very easily raised on handling unless great care is exercised not to excite the animal. It is necessary to ensure that the rabbit is perfectly comfortable during the taking of blood samples. The ear vein from which samples are to be drawn is pierced some hours before blood is required for sugar estimations, so that frequent small samples can be obtained without the slightest reaction on the part of the rabbit.

In most instances the blood sugar curves given in the present paper represent the average response for 6 or more rabbits. Average, rather than individual, results are given because small individual variations are thereby minimized. The groups of animals for which average curves are presented sometimes constituted only a small proportion of the total number of rabbits used in a particular type

of experiment, and the individual experiments for which average results are given were chosen at random from the total number available. Where the difference between the average curves for two different groups might or might not be significant, standard errors of corresponding values in the average curves were calculated. In this way it was possible to determine whether or not corresponding points in the two average curves differed significantly.

Adrenaline infusion

Adrenaline was infused into the marginal ear vein of the unanaesthetized rabbit following the technique of Cori *et al.* [1930], and using a Burn-Dale burette pump. 1/1000 adrenaline (Parke Davis Ltd.) was diluted to a suitable volume with 0·9 % NaCl solution containing 1 mg. of glutathione/5 ml., the latter being added to stabilize the dilute solution of adrenaline.

Anterior pituitary extracts

Extracts of ox, pig and horse pituitary glands were used in the present investigation. Most of the extracts were obtained from ox pituitary gland, however, and where the species-source is not stated it is to be assumed that the preparation was extracted from ox anterior lobe.

All the ox pituitary preparations were extracted from anterior lobe tissue which had been dissected as free as possible from posterior lobe tissue. In a number of instances, however, extracts of pig and horse pituitary tissues were made from the undissected glands.

(*a*) *Acetone desiccation of anterior lobe tissue.* Acetone desiccation of frozen ox anterior lobes was effected by mincing the frozen tissue into 20 vol. of ice-cold acetone. After remaining in acetone in the cold room overnight, the tissue was transferred at intervals of 24 hr. to 3 or 4 fresh portions of cold acetone. The dehydrated tissue was finally washed with ether and preserved in a vacuum desiccator at room temperature. Frozen pig or horse pituitary tissue was desiccated in acetone at room temperature. Commercial (B.D.H. Ltd.), acetone-desiccated ox anterior lobe tissue was used for a number of preparations.

(*b*) *Preparation of prolactin.* Six different types of prolactin were used. These differed in the source (ox, horse or sheep pituitaries; fresh gland or acetone-desiccated powder) and in the methods of extraction. The different types are distinguished by suffixes.

Prolactin (1) was obtained from fresh, frozen dissected glands by the method described by Young [1938]. The material designated Prolactin-C in the earlier paper is the Prolactin (1) of the present communication. In brief, the method of preparation consisted of alkaline extraction of the anterior lobe tissue at 0°, followed by precipitation and reprecipitation of the prolactin at *p*H 5·5. All these procedures were carried out in the cold room at 0° as far as possible, and the prolactin was stored in a frozen condition at −11°, in a solution of such concentration that 3 ml. were equivalent to 1 g. of fresh anterior lobe tissue.

Prolactin (2) was prepared from commercial acetone-desiccated ox anterior lobe by the method of Bates & Riddle [1935] using either 60 % or 70 % alcohol for the initial extraction, though a slight addition to the Bates-Riddle technique was made. This consisted in a number of reprecipitations from water at *p*H 5·5 of the material insoluble at *p*H 3·5 in the presence of Na_2SO_4. In this way any Na_2SO_4 present in the original precipitate was removed. The product was then dried and passed through the final stage of the Bates-Riddle method of purification.

Prolactin (3) was prepared from acetone-desiccated fresh gland by a method essentially that of Riddle *et al.* [1933], i.e. aqueous extraction at *p*H 8 followed by precipitation (and reprecipitation a large number of times) of the prolactin fraction at *p*H 5·5. The prolactin was finally washed with alcohol and ether and dried.

Prolactin (4) was similar to Prolactin (3) except that it was extracted from commercial dried gland.

Prolactin (5) was prepared by the slight modification of the Bates-Riddle method described by Young [1938]. The modification consisted in the replacement of the final fractionation with 70 % alcohol in the presence of NaCl by 6–12 reprecipitations from a large volume of water at *p*H 5·5. The product was washed with alcohol and ether and dried.

Prolactin (6) was the product obtained by an application to Prolactin (5) of a method of purification based on that of Lyons [1937]. 1 g. Prolactin (5) was stirred for 1 hr. at room temperature with 40 ml. *N*/20 HCl contained in 80 % aqueous acetone; the residue was extracted twice in the same manner. The combined extracts were then brought to the point of maximum precipitation by the addition of strong ammonia solution (about 0·25 ml.) and two volumes of alcohol were added. After settling overnight the precipitate was filtered off and dissolved in water at *p*H 8, any material insoluble at this *p*H being spun off and discarded, and it was then reprecipitated at *p*H 5·5. This process of solution at *p*H 8 and precipitation at *p*H 5·5 was repeated, and the precipitated prolactin was washed with alcohol and ether and dried.

(*c*) *Preparation of thyrotropic extracts.* The material which remained in solution when the prolactin fraction from ox pituitary tissue had been precipitated "isoelectrically" during the preparation of Prolactins (1), (2), (3) and (4) constituted the thyrotropic fractions used in the present investigation. No attempt was made to separate the thyrotropic and gonadotropic hormones, and all the thyrotropic preparations used possessed gonadotropic activity.

In each instance a thyrotropic preparation is numbered in agreement with the corresponding prolactin preparation.

Thyrotropic (1), i.e. the *p*H 5·5-soluble material corresponding with Prolactin (1), is the fraction described by Young [1938] as "non-prolactin-C". This was not precipitated and dried, but stored in solution by freezing at −11°. The solution was of such strength that 3 ml. of it were equivalent to 1 g. of fresh gland tissue.

Thyrotropic (2), the thyrotropic fraction corresponding with Prolactin (2), was prepared and dried according to the method previously described [Young, 1938]. This fraction was designated "ordinary non-prolactin" in the previous paper.

Thyrotropic (3) and *Thyrotropic* (4) were precipitated by the addition of 6 vol. of cold alcohol, washed with alcohol and ether and dried *in vacuo*.

(*d*) *Average yields of the different fractions.* The yields of the different types of preparation used are given in Table I as percentages of acetone-desiccated anterior pituitary tissue.

(*e*) *Posterior lobe contamination of anterior pituitary preparations.* As the administration of posterior pituitary extracts is known to affect carbohydrate metabolism, it was necessary to be certain that the anterior pituitary preparations used were not grossly contaminated with posterior lobe principles. The results of assays for oxytocic activity (guinea-pig uterus method) on the different preparations used are given in Table II. In a few instances the pressor activity of the ox pituitary preparations was determined, and found to be close to the figure expected from the previously determined oxytocic activity.

Table I. *Yields of the different pituitary preparations used*

Source of gland	Method of preparation	Yield, expressed as % of desiccated anterior lobe: Prolactin fraction	Thyrotropic fraction
Ox	(1)	—*	—*
	(2)	1·0– 1·6	0·8–1·3
	(3)	9·8–11·1	2·2–2·7
	(4)	5·3– 9·3	2·2–3·0
	(5)	6·7–12·8	—
	(6)	About 2	—
Horse	(3)	8·0–12·0†	—
Pig	(3)	11·7–14·2†	—

* These fractions were not dried before use but stored frozen in solution.
† Yield expressed as % of whole pituitary gland.

Table II. *Oxytocic activity of pituitary preparations*

(The preparations were heated in a boiling water bath with 0·25% acetic acid for 2 min. before assay.)

Source of gland	Method of preparation	Oxytocic activity (international units): Prolactin fraction	Thyrotropic fraction
Ox	(1)	About 0·005 units/ml.	About 0·006 units/ml.
	(2)	<0·0001 units/mg.	0·25–0·75 units/mg.
	(3)	<0·0001 ,,	<0·0001 ,,
	(5)	<0·0001 ,,	—
	(6)	<0·0001 ,,	—
Horse	(3)	About 0·0003 ,,	—
Pig	(3)	About 0·0001 ,,	—

The results in Table II show that with the exception of Thyrotropic (2) the ox pituitary preparations used possessed negligible oxytocic activity.

Results

1. *The influence of pituitary extracts on the hypoglycaemic action of insulin*

In many early experiments of the present investigation on the glycotropic action of anterior pituitary extracts, 2 injections of a suitable extract were given daily to a rabbit for 2–4 days, and the response to 2 units of crystalline insulin, injected intravenously after a fast of 21 hr., was determined on the day following the last injection of extract. In these experiments the blood sugar curve following the administration of insulin was usually of the form illustrated in Fig. 1 [cf. Young, 1936, 1]. Occasionally, however, the daily injection of the anterior pituitary extract itself raised the blood sugar level of the rabbit [cf. Young, 1938] so that on the 3rd or 4th day of injection the blood sugar was at a definitely diabetic level. In these instances a glycaemic response to the injection of insulin, somewhat resembling that illustrated in Fig. 1, was sometimes, but not always, obtained (Fig. 2). In later experiments only two injections of anterior pituitary extract were given, these being administered at the beginning and at the 16th hour of a short fast. Two units of crystalline insulin were injected intravenously at the beginning of the 21st hour of fasting, and the blood sugar level was determined at intervals. With suitable pituitary extracts the hypoglycaemic action of the insulin was completely abolished (Fig. 3), there being no sign of an

initial, transient hypoglycaemia. Indeed, in most instances the immediate effect of the insulin injection was a definite rise of blood sugar level to 3–5 mg./100 ml. above its original level. Such a rise was not present when a volume of saline equal to that of the insulin solution was injected intravenously into the pituitary-treated rabbits. It should be mentioned that in spite of the fact that crystalline insulin (Burroughs Welcome) was used, a slight initial hyperglycaemic response to the intravenous injection of insulin was frequently observed in the control experiments on rabbits which had not been previously treated with pituitary extracts.

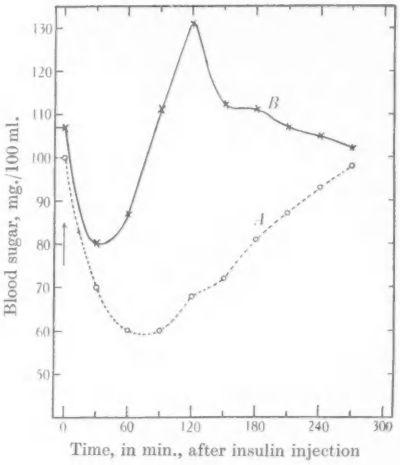

Fig. 1.

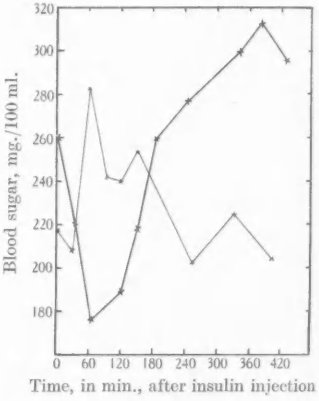

Fig. 2.

Fig. 1. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A*. Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 7 normal rabbits which had fasted 18 hr. *Curve B*. Average blood sugar response to the same dose of insulin of the same rabbits after each had received 4 injections of glycotropic substance during 2 days.

Fig. 2. The response to the intravenous injection of 2 units of crystalline insulin of two rabbits which exhibited hyperglycaemia as the result of treatment with the glycotropic substance.

It should be pointed out that the experiments which yielded curves of the type shown in Fig. 1 were those in which the injections of the glycotropic substance were given over a period of some days, during which time the rabbits were feeding normally. Curves of the type shown in Fig. 3 were obtained in experiments with rabbits which received pituitary injections during a short period of fasting, but it is not clear how far the condition of the animal (fasting or fed) at the time the injections of pituitary extract were given, determined the type of response to insulin.

In all the above experiments insulin was administered intravenously, but it should be mentioned that similar results were obtained when insulin was injected subcutaneously [cf. Marks & Young, 1938].

Control experiments with extracts of muscle, liver, adrenal and pineal tissues, prepared in a manner similar to that for the extraction of pituitary gland, showed no detectable glycotropic activity in these tissues, under the conditions of our experiments (Fig. 4—Curve A).

Rabbits treated with glycotropic pituitary extracts have more liver glycogen at the end of a short fast than do control animals [Young, 1937, 2; Marks & Young, 1938]. Control experiments were therefore made on rabbits in which the fasting liver glycogen level was raised. It was found that if our rabbits were given

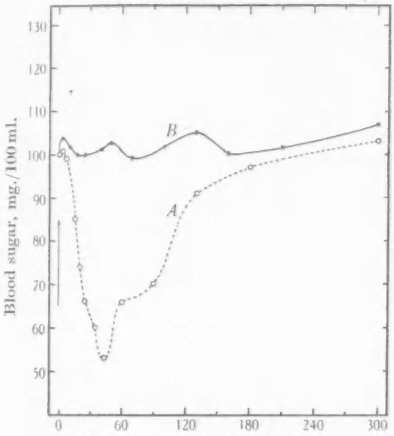

Fig. 3.

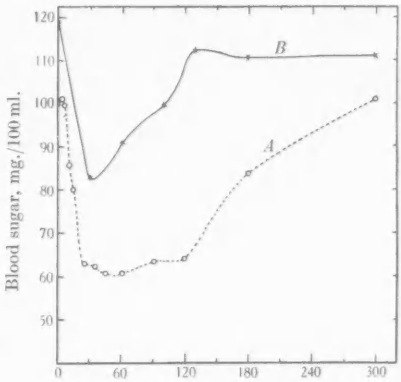

Fig. 4.

Fig. 3. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A*. Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 normal rabbits which had fasted 21 hr. *Curve B*. A typical response to the intravenous injection of 2 units of crystalline insulin of a rabbit which had received 2 injections of glycotropic substance during the preliminary fast of 21 hr.

(In order to facilitate comparison, the blood sugar results are expressed as a percentage of the initial level. In the control group the initial level was 103 mg./100 ml. while in the treated animal it was 106 mg./100 ml.)

Fig. 4. The influence of treatment with non-specific tissue extracts and of feeding with glucose, on the hypoglycaemic action of insulin in the rabbit. *Curve A*. Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 rabbits which had received 2 injections of various tissue extracts during the preliminary fast of 21 hr. *Curve B*. Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 18 rabbits which had received 24 g. of glucose by mouth at the beginning of the preliminary fast of 21 hr.

24 g. of glucose in 40 ml. of water by stomach tube at the beginning of a 21 hr. fast, the liver glycogen at the end of the fast averaged 3·2 % as compared with 1·8 % for untreated rabbits, and 5·1 % for rabbits treated with glycotropic substance (Prolactin (1)) during the period of fasting. The blood sugar level of the glucose-fed rabbits was slightly, but significantly, raised, as the average initial blood sugar in 18 experiments was $118{\cdot}0 \pm 2{\cdot}4$ mg./100 ml.,[1] compared with a normal value (50 animals) of $103{\cdot}6 \pm 2{\cdot}3$. However, a comparison of Curve A,

[1] 2·4 is the standard error of the mean value 118·0. The standard error of the mean is calculated from the formula $\epsilon = \sqrt{\frac{\epsilon(\bar{x}-x)^2}{n(n-1)}}$ where $\bar{x}$ = the mean value of the series of observations, x = any individual observation, and n = the number of observations. The difference between two mean values is taken to be significant if the value of $\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$ is greater than 3.

Fig. 3, with Curve B, Fig. 4, shows that in these animals the hypoglycaemic action of insulin was but little altered.

When the glycotropic activity of pituitary extracts was tested according to the later technique described above, the blood sugar level of the rabbits was never more than very slightly raised by the injections of the pituitary extracts alone, provided that the animals had been handled very carefully, and the extracts were of a suitable type. In 50 such experiments with active preparations, the average blood sugar level immediately before the insulin injection was $107{\cdot}4 \pm 2{\cdot}4$ mg./100 ml. while in 50 untreated control animals, which had likewise fasted for 21 hr., the blood sugar level averaged $103{\cdot}6 \pm 2{\cdot}3$. These results show that the administration of the anterior lobe extracts alone had no significant effect on the fasting blood sugar level of rabbits under the conditions of these experiments.

In order to facilitate the comparison of the glycotropic activities of different pituitary preparations, it was desirable to adopt a "unit" of glycotropic activity. As rabbits appear to be somewhat less sensitive to the anti-insulin action of glycotropic extracts during the middle of the summer, the results of assays carried out during the winter months only were used in this connexion. A unit of glycotropic activity was defined as that amount of extract which, on subcutaneous administration to a fasting rabbit in two equal doses according to the method described above, would just abolish, or almost abolish, the hypoglycaemic action of 2 units of crystalline insulin administered intravenously after a fast of 21 hr. The hypoglycaemic action of the insulin was considered to be almost abolished if the blood sugar level failed to fall more than 10 mg./100 ml. below its original value within 3 hr. of the insulin injection. An approximate value for the minimum amount of extract required could usually be determined in 3–5 experiments. Although no great quantitative merit can be claimed for the results of these assays, the differences observed between the activities of different types of extract were of such a magnitude as to be demonstrable by this method.

The evidence that prolactin and the glycotropic substance are different springs from two main sources: (*a*) a consideration of the widely differing glycotropic activities of different prolactin preparations obtained by different methods from pituitary glands of the same species, and by the same method from pituitary glands of different species; (*b*) the preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.

(*a*) *The relative glycotropic activities of different preparations of prolactin.* Table III gives the average prolactin and glycotropic activities of different prolactin preparations. It will be seen that Prolactin (6), although highly active as a pigeon crop gland stimulator, possessed no detectable glycotropic activity. On the other hand, Prolactin (1), extracted from pituitary glands of the same

Table III. *Average prolactin and glycotropic activities of prolactin preparations obtained by different methods*

Source of hypophysis	Method of extraction	Prolactin content Riddle units	Glycotropic activity units	Prolactin units / Glycotropic units
Ox	(1)	15/ml.	8/100 ml.	190
	(2)	6/mg.	12/g.	500
	(3)	3·5/mg.	6/g.	580
	(4)	0·5/mg.	1/g.	500
	(5)	3·5/mg.	5/g.	700
	(6)	6/mg.	<1/g.	>6000
Horse	(3)	0·2/mg.	12/g.	17
Pig	(3)	0·3/mg.	6/g.	50

species, possessed substantial glycotropic activity. Horse and pig pituitary tissues are poor in prolactin activity, but possess glycotropic activity comparable with that of ox pituitary tissue. It will be seen from Table III that the maximum value of the ratio prolactin activity/glycotropic activity (6000) is over 350 times the minimum value (17), which shows that there can be no relationship between the pigeon crop gland activity and glycotropic activity, i.e. prolactin and the glycotropic factor cannot be identical.

From the data in Tables II and III one can calculate the total prolactin and glycotropic activities of 100 g. of acetone-desiccated ox pituitary gland, as indicated by the results for the different preparations. The results obtained, which are given in Table IV, enable a comparison to be made of the different proportions of the total activities present in the desiccated gland which are carried through into these preparations. Prolactin preparations (1), (3) and (5) contain similar proportions of the total prolactin activity present in the gland tissue, but Prolactin (1) contains at least twice the total glycotropic activity of the other preparations. Prolactin (1) is obtained by a method involving only one extraction of the anterior lobe tissue [Young, 1938], so that a loss of active material must occur in the primary extraction. This probably accounts for the lower proportion of the total crop gland-stimulating material found in Prolactin (1), but renders the greater proportion of glycotropic activity found in this fraction more striking.

Table IV. *Prolactin and glycotropic activities contained in 100 g. of acetone-desiccated ox anterior lobe, as calculated from the data for the different specimens of prolactin*

	100 g. of ox acetone-desiccated gland calculated to contain	
Method of extraction	Prolactin Riddle units	Glycotropic activity units
(1)	22,500*	120*
(2)	7,800	16
(3)	36,400	63
(4)	3,650	7
(5)	34,000	49
(6)	12,000	<2

* Calculated on the assumption that 5 g. of fresh tissue = 1 g. of acetone-desiccated gland.

(*b*) *The preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.* A number of preparations of the thyrotropic hormone from ox pituitary gland were found to possess substantial glycotropic activity. The relevant data are summarized in Table V.

Table V. *Glycotropic activity of preparations of the thyrotropic hormone*

(All preparations extracted from ox hypophysis.)

Method of preparation	Thyrotropic activity Rowlands-Parkes units	Glycotropic activity units	Prolactin activity Riddle units
(1)	2/ml.	5/100 ml.	<0·3/ml.
(2)	1·5/mg.	<1/g.*	<0·03/mg.
(3)	1·0/mg.	5/g.	—
(4)	0·3/mg.	<1/g.*	—

* These preparations were contaminated with posterior lobe hormones. The results of the glycotropic tests are therefore unreliable (see below).

As preparations of the thyrotropic hormone which contain no amount of prolactin detectable by our methods possess substantial glycotropic activity, it is

again emphasized that prolactin and the glycotropic substance cannot be identical.

(c) *Differentiation of the glycotropic substance from the thyrotropic and gonadotropic hormones.* A number of the prolactin preparations which possessed glycotropic activity contained only traces of the thyrotropic and gonadotropic hormones. In particular Prolactin (2) and Prolactin (5) possessed so little thyrotropic activity that the administration of 20 mg. daily for 5 days failed to induce any enlargement of the thyroid gland of the immature guinea-pig under the conditions of our test. Similarly, the intravenous administration of 100 mg. of preparations of these types to an oestrous rabbit failed to cause ovulation. The amounts of thyrotropic and gonadotropic hormones contaminating these prolactin preparations must therefore have been very small, although their glycotropic activities were substantial. It is clear that there is no relationship between the gonadotropic and thyrotropic activities of pituitary extracts and their glycotropic activity, so that the glycotropic substance cannot be identical with the gonadotropic and thyrotropic hormones.

(d) *Differentiation of the glycotropic substance from the oxytocic and pressor substances of the posterior pituitary gland.* As seen above, anterior lobe extracts which contain only a trace of the oxytocic substance possess substantial glycotropic activity. Fig. 5 gives the average blood sugar response to 2 units of

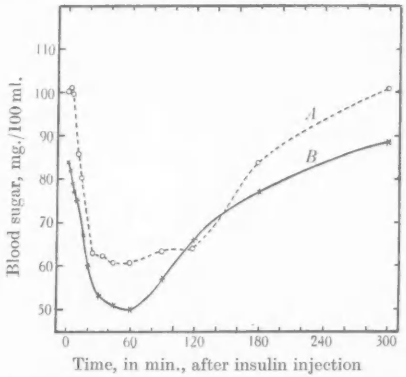

Fig. 5. The influence of treatment with pituitrin on the hypoglycaemic action of insulin in the rabbit. *Curve B.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 6 rabbits each of which had received 2 injections of 30 units of pituitrin (Parke Davis) during the preliminary fast of 21 hr.

Curve A is the control curve from Fig. 4, which is inserted for the purpose of comparison.

crystalline insulin in a group of 6 rabbits, each of which received 2 injections of pituitrin (pressor + oxytocic substance—Parke Davis) containing together 60 units of oxytocic activity (Curve B). The average response to the same dose of insulin in control experiments (Curve A, Fig. 4) is included for comparison. The initial blood sugar level of the animals treated with posterior lobe extract was usually slightly below that of control animals (average value in 6 experiments = 84·0 $\pm$ 5·3), but the hypoglycaemic action of insulin was normal, the blood sugar level falling to about 60 % of its initial value in both groups of rabbits.

Burn [1923] found that when a posterior lobe extract and insulin were simultaneously injected subcutaneously into a rabbit, the hypoglycaemic action

of the insulin was almost abolished. Many other investigators have also found that pituitrin administration inhibits the hypoglycaemic action of subcutaneously administered insulin and depresses the sugar tolerance [cf. Cohen & Libman, 1937]. As Lambie & Redhead [1929] point out, the circulatory changes produced by the injection of pituitrin are profound and these may greatly modify the rate of diffusion into the tissues of injected material. The depressed rate of absorption of subcutaneously administered insulin may possibly account partly for the inhibitory action of simultaneously administered pituitrin. In the present investigation the second dose of pituitrin was given some hours before the intravenously injected insulin, and it is clear that the results of these experiments are not directly comparable with those of Burn and others. Our results do show, however, that the anti-insulin activity of the anterior lobe extracts was not due to the presence of traces of the oxytocic and vasopressor substances of the posterior lobe.

Anterior lobe extracts heavily contaminated with the oxytocic and vasopressor substances have always failed to exhibit glycotropic activity, even when there was reason to suspect that glycotropic substance was present in the extract. In a number of instances it was also found that the glycotropic activity of anterior lobe preparations was diminished when posterior lobe extracts were added to them. All these results are explicable on the assumption that when the anterior lobe extracts are heavily contaminated with the oxytocic and vasopressor substances, the diminished rate of absorption of the anterior lobe extracts caused by the intense vasoconstriction decreases the effectiveness of the administered glycotropic substance.

2. *The influence of injections of the glycotropic factor on the sugar tolerance curve in the rabbit*

It was briefly reported last year [Young, 1937, 1] that in a rabbit which has received 2 injections of a suitable preparation of the glycotropic substance and which has thereby become insensitive to the hypoglycaemic action of 2 units of insulin, the sugar tolerance curve is not significantly affected. Fig. 6 gives the

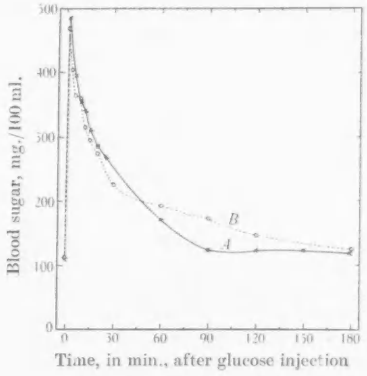

Fig. 6. The influence of treatment with the glycotropic substance on the sugar tolerance curve in the rabbit. *Curve A*. The average blood sugar response to the intravenous injection of 2 g. of glucose in 10 ml. of water of 6 normal rabbits which had fasted 21 hr. *Curve B*. The average blood sugar response to the same intravenous dose of glucose of the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

average blood sugar response of a group of 6 rabbits which had fasted 21 hr. to the intravenous injection of 2 g. of glucose in 10 ml. of water, administered at 0 min. (Curve A). Curve B is the average response of the same group after each rabbit had received 2 injections of a glycotropic pituitary extract (in most instances Prolactin (1)) which contained in all 1 unit of glycotropic activity. The blood sugar response to the intravenous administration of 2 g. of glucose is remarkably little affected by previous treatment with an amount of glycotropic substance which induces complete insensitivity to the hypoglycaemic action of insulin. For the first hour after the injection of glucose there is practically no difference between the average fall of blood sugar level in the control group of animals and that in the group treated with the glycotropic extract. The blood sugar in the pituitary-treated rabbits does not reach quite such a high level as it does in the control animals, and appears to fall a little more quickly for the first 60 min., though no difference between the two curves is statistically demonstrable. After 60 min. the blood sugar level in the control group falls faster than that of the experimental group so that at 90 min. a difference exists between the two curves which is possibly statistically significant $\left(\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}} = 2{\cdot}6\right)$. The subnormal rate of fall of blood sugar in the experimental group, beginning about one hour after the glucose injection, could be explained in two ways. First, the glycogen stores of a rabbit which has received injections of a glycotropic extract during a 21 hr. fast are considerably higher than those of control animals [Young, 1937, 1]. It is therefore possible that one hour after the injection of glucose the glycogen stores of the pituitary-treated animals are so full that a diminution in the rate of glycogen storage occurs with a consequent decreased rate of removal of sugar from the blood. Second, as Fisher *et al.* [1936] have shown, pituitary extracts may exert a depressing action on carbohydrate oxidation during a fast. If, in the normal animal, the fall of blood sugar for about one hour after glucose is predominantly due to storage of glycogen, while the subsequent fall is primarily associated with carbohydrate oxidation, then the observed facts would find an explanation, though it must be confessed that at present there seems little evidence to support such a theory.

If a crude saline extract of ox anterior lobe is used as the source of glycotropic activity, the sugar tolerance of the injected rabbit may be slightly, though definitely, decreased. This is also true for a number of cases in which Thyrotropic (1) is used, but does not detract from the value of the observation that the administration of other preparations of glycotropic factor, in a dose which is sufficient to abolish the hypoglycaemic action of 2 units of insulin, has very little effect on the sugar tolerance curve. It is probably of significance that those extracts (crude saline extract and Thyrotropic (2)) which are sometimes found to diminish the sugar tolerance in the rabbit under the conditions of the above test, are those which were found to be diabetogenic in the dog [Young, 1938].

Himsworth & Scott [1938, 1] have recently investigated the influence of various conditions on the increase in sugar tolerance which occurs in the rabbit receiving a high carbohydrate diet as the result of consecutive intravenous injections of 1 g. of glucose at intervals of half-an-hour (Staub-Traugott effect). They found that the hypophysectomized rabbit receiving a high carbohydrate diet showed a Staub-Traugott effect, in that each successive injection of glucose resulted in a smaller rise of blood sugar level than the previous one. When the hypophysectomized rabbit received preliminary treatment with a preparation of the glycotropic substance, the sugar tolerance was diminished, as shown by the absence of Staub-Traugott effect when successive injections of glucose were

given. The impairment of sugar tolerance was similar to that which occurs when a normal rabbit receives a diet low in carbohydrate. In view of the results of Himsworth & Scott with hypophysectomized rabbits it was necessary to determine whether or not the Staub-Traugott effect was abolished in an intact rabbit treated with the glycotropic substance. Although extensive investigations have not been carried out on this point, the results show that, as with hypophysectomized rabbits, the Staub-Traugott effect is abolished in normal rabbits by treatment with the glycotropic substance (Fig. 7). This decrease of tolerance,

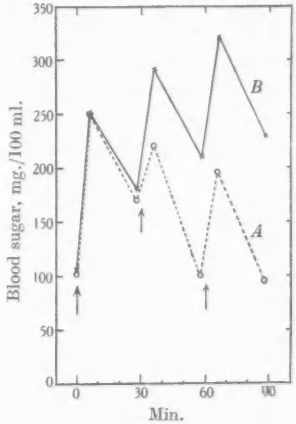

Fig. 7. The influence of treatment with the glycotropic substance on the sugar tolerance in the rabbit. *Curve A*. The blood sugar response to three intravenous injections of 1 g. of glucose, administered at half-hourly intervals to a normal rabbit. *Curve B*. The blood sugar response in the same rabbit after two injections of the glycotropic substance. 1 g. of glucose in 10 ml. of water was injected at the times indicated by the arrows.

which is explicable on the basis of either of the suggestions put forward above, is similar to that which occurs in rabbits fed on a low carbohydrate diet [Himsworth & Scott, 1938, 1]. The sugar tolerance of rabbits which have been rendered completely insensitive to the hypoglycaemic action of injection of insulin by treatment with the glycotropic substance, is therefore only very slightly decreased, and remains within the range of variation to be expected in normal rabbits receiving different diets.

3. *The influence of injections of the glycotropic factor on the hyperglycaemic response to adrenaline in the rabbit*

Corkill *et al.* [1933] and Cope & Marks [1934] found that in the rabbit hypophysectomy greatly decreased the hyperglycaemic action of subcutaneously administered adrenaline, and conversely, that the administration of a crude anterior lobe extract greatly increased the blood sugar response to the subcutaneous injection of adrenaline. Administration of a suitable preparation of prolactin to the normal rabbit also greatly increases the glycaemic response to the subcutaneous injection of adrenaline [Young, 1936, 1, 2].

Curve A (Fig. 8) gives the average hyperglycaemic effect of the subcutaneous injection of 20μg. of adrenaline in a group of 10 normal rabbits which had fasted for 21 hr. Curve B (Fig. 8) is the average response of the same group to the same

dose of subcutaneous adrenaline after each rabbit had received two injections of prolactin (totalling 1 unit of glycotropic activity/rabbit) during the preliminary period of fasting. The difference in average response in the two groups is statistically significant, e.g. at 120 min. the average blood sugar level of the control group was $121 \pm 3{\cdot}5$ mg./100 ml. while that for the experimental group was 165 ± 14; similar results were obtained for other points in the middle of the curves. An increase in the hyperglycaemic response to the subcutaneous injection of adrenaline was also found in a rabbit which had received injections of the glycotropic substance twice daily for 2–4 days.

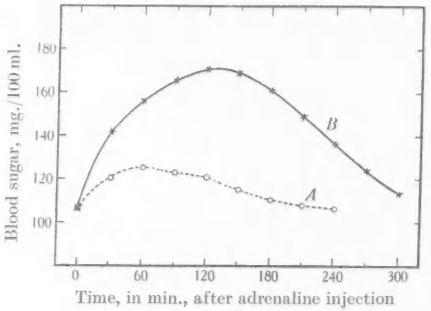

Fig. 8.

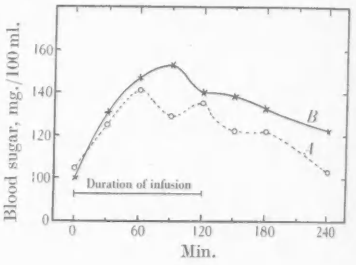

Fig. 9.

Fig. 8. The influence of treatment with the glycotropic substance on the hyperglycaemic action of subcutaneously administered adrenaline in the rabbit. *Curve A*. The average blood sugar response to the subcutaneous administration of 20 μg. of adrenaline in 10 normal rabbits which had fasted 21 hr. *Curve B*. The average blood sugar response to the same subcutaneous dose of adrenaline in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

Fig. 9. The influence of treatment with the glycotropic substance on the hyperglycaemic response to the intravenous infusion of adrenaline in the rabbit. *Curve A*. The average blood sugar response to the intravenous infusion of adrenaline at the rate of 0·05 μg./kg. body weight/min., for 2 hr., in 7 normal rabbits which had fasted for 21 hr. *Curve B*. The average blood sugar response to the intravenous infusion of adrenaline at the same rate in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

As stated above, Corkill *et al.* [1933] and Cope & Marks [1934] found that the hyperglycaemic effect of subcutaneously administered adrenaline was greatly diminished in hypophysectomized rabbits. Russell & Cori [1937] have recently shown that the effect of the administration of adrenaline on the blood sugar level of the anaesthetized hypophysectomized rat was somewhat diminished when the adrenaline was given subcutaneously, but was normal when the adrenaline was given by slow intravenous infusion. These authors suggest that hypophysectomy diminishes the rate of absorption of adrenaline from the subcutaneous tissues of the rat. A comparison of the hyperglycaemic action in the normal rabbit treated with glycotropic substance, of adrenaline administered subcutaneously and by slow intravenous infusion, was therefore of interest.

Fig. 9 gives the average result for seven experiments in which a normal rabbit which had fasted 21 hr. received a slow intravenous infusion of adrenaline for 120 min. at the rate of 0·05 μg./min./kg. body weight (curve A), and the average response to adrenaline infused for the same time and at the same rate in the same group, when each animal had received two injections of Prolactin (1), containing altogether 1 unit of glycotropic activity, during the preliminary fast (Curve B).

The slight differences between corresponding points on these two curves are not statistically significant. The results show, therefore, that although the hyperglycaemic response to subcutaneously administered adrenaline is enhanced in the rabbit treated with the glycotropic substance, the response to the slow intravenous infusion of adrenaline was, under the conditions of our experiments, unchanged within the limits of experimental error.

As hypophysectomy diminishes the rate of absorption from the subcutaneous tissues [Russell & Cori, 1937; Heinbecker & Weichselbaum, 1937], it is possible that the administration of pituitary extracts also influences the rate of subcutaneous absorption. According to Heinbecker [1937] removal of the pituitary gland increases the action of adrenaline on the smooth muscle of the cat, and Heinbecker & Weichselbaum [1937] suggest that in the hypophysectomized animal adrenaline would cause an accentuated contraction of the blood vessels at the site of the subcutaneously injected adrenaline, with a consequent diminution in the rate of absorption. These authors consider that this diminution may account for the decreased hyperglycaemic action of subcutaneously administered adrenaline. If the injection of our pituitary extracts has an action on the response of smooth muscle to adrenaline, which is the converse of that of hypophysectomy, then the administration of glycotropic pituitary preparations might be expected to increase the rate of absorption of adrenaline from the subcutaneous tissues. The curves in Fig. 8 show that the action of subcutaneous adrenaline on the blood sugar is more prolonged in the pituitary-injected animals, and it would seem impossible to explain this effect by a more rapid absorption of adrenaline from the subcutaneous tissues. At present there seems to be no obvious explanation as to why the hyperglycaemic effect of subcutaneously injected adrenaline is so greatly enhanced in the pituitary-treated animals. The slight increase in response to the intravenous infusion of adrenaline in the pituitary-treated animals, although not statistically significant on the basis of the number of experiments carried out, may nevertheless indicate a slightly greater sensitivity to the hyperglycaemic action of adrenaline which has reached the blood stream, as the result of treatment with the glycotropic substance. As the treated rabbits have more glycogen in their livers at the end of the preliminary fasting period than do control animals [Young, 1937, 2] a slight increase in sensitivity to the hyperglycaemic action of adrenaline would be explicable on this basis alone, without recourse to a hypothesis that the liver glycogen becomes more easily mobilized under the influence of the pituitary extract [cf. Cope & Marks, 1934].

It should be recorded in this connexion that in our experience rabbits treated with the glycotropic substance are very liable to exhibit hyperglycaemia as the result of a slight emotional stimulus. No special investigation has been made on this point, and it is impossible to say whether or not observations of this kind would be found consistently in all rabbits, but it has become increasingly clear during a long series of experiments with the glycotropic substance, that in general, careful handling of the pituitary-treated animals is required if the blood sugar is to remain at its normal level. It is conceivable that the hyperglycaemic action of nerve stimuli is enhanced in these animals. The action of sympathin on the blood sugar has recently been investigated by Bodo & Benaglia [1938], but the action of pituitary extracts on the degree of hyperglycaemia following nerve stimulation in animals with denervated adrenal glands, has apparently not yet been investigated.

Collip [1938] finds that the injection of his pituitary anti-insulin substance inhibits the hyperglycaemic response to injected adrenaline. In our experiments there has been no indication of an inhibition of adrenaline hyperglycaemia in the pituitary-treated rabbits.

Discussion

The results of the present investigation confirm the assumption that the pituitary gland contains a substance, the injection of which does not significantly alter the blood sugar of the fasting rabbit under the conditions of our experiments, but induces a complete insensitivity to the hypoglycaemic action of insulin. It was previously found that the daily injection of large doses of this "anti-insulin" substance into normal dogs did not induce glycosuria or ketonuria and that the "diabetogenic" and "anti-insulin" principles were therefore not identical [Young, 1936, 1, 2; 1938].

The name "glycotropic factor" was suggested as a convenient one for the pituitary substance which antagonizes the hypoglycaemic action of insulin without itself inducing a hyperglycaemia [Young, 1936, 2]. The term "glycotropic" was not intended to imply any specific mechanism of action of the "anti-insulin" factor, although at the time when the name was suggested it was believed that one action of the glycotropic substance was a facilitation of hepatic glycogenolysis, particularly the glycogenolytic action of adrenaline. Even at that time it was realized that this view was not altogether satisfactory [Young, 1936, 1], and the results since obtained do not support it. At present the mechanism whereby the anti-insulin action is exerted is not clear (see below), but until more is known about the nature and mechanism of action of the principle concerned, it is proposed to retain the name "glycotropic" factor or substance to describe it.

The identity of the glycotropic substance

The results of the present investigation differentiate the glycotropic substance from prolactin (pigeon crop gland-stimulating substance), the thyrotropic and gonadotropic hormones, and the oxytocic and pressor substances of the pituitary gland. Tissues other than the pituitary gland have failed to yield active extracts. So far there is no evidence as to whether or not the glycotropic substance is identical with the adrenotropic, ketogenic or glycostatic [Russell & Bennett, 1936] pituitary substances, or with the pituitary substance which increases the fat content of the liver [Best & Campbell, 1936].

According to a recent preliminary communication by Collip [1938], the pituitary melanophore-expanding substance, the ketogenic and anti-insulin substances, and a pituitary substance which antagonizes the hyperglycaemic action of adrenaline, are all identical. The single principle responsible for all these effects is believed to be produced in the pars intermedia of the pituitary gland. The full evidence for the belief that only one principle is responsible for so many different effects is not yet available. The results of the present investigation throw no light on the question whether or not the glycotropic substance is identical with the substance or substances responsible for the effects considered by Collip, and a discussion of this question is therefore not profitable at present.

In our experience loss of activity always occurs when preparations of the glycotropic substance are dried, and, as shown in Tables III and IV, Prolactin (1), which is an undried extract, contains a much higher proportion of glycotropic activity than the other preparations used. Our extracts containing the glycotropic substance can be dialyzed in cellophane bags (Visking) without undergoing appreciable loss of activity, but Collip [1938] finds that a substance capable of neutralizing the hypoglycaemic action of insulin will pass through a cellophane or collodion membrane, if the solution has been previously adjusted to *p*H 10 and heated on a boiling water bath for one hour. Further investigations on the

stability of the glycotropic substance towards heating in alkaline solution are required before the relation between the results of the present investigation and Collip's interesting findings is clear.

The most active preparations of the glycotropic substance were obtained from anterior lobe which had been carefully dissected from posterior lobe tissue, so that these preparations were virtually free from oxytocic activity (Table II). It is therefore safe to assume that the glycotropic substance is present in either the pars anterior or the pars intermedia, both of which may be present in the anterior lobe as obtained by our method of dissection. Collip [1938] believes that his anti-insulin substance is formed in the pars intermedia, but at present no evidence is available to determine whether or not this is true for the glycotropic substance.

The mechanism of action of the glycotropic substance

Cope & Marks [1934] found that in hypophysectomized rabbits the blood sugar often fell to a very low level and the animals exhibited symptoms of hypoglycaemia in spite of the presence of ample reserves of liver glycogen. They also found that the hyperglycaemic action of subcutaneously injected adrenaline was decreased in hypophysectomized rabbits and increased in rabbits treated with suitable anterior lobe extracts. These authors therefore put forward the attractive hypothesis that the anterior lobe of the pituitary gland contains a principle which renders the glycogen stores of the liver more susceptible to the mobilizing action of adrenaline [cf. also Young, 1936, 1]. More recently Cope [1937] has found that in the fasting hypophysectomized young rabbit, the blood sugar level is maintained until the liver glycogen stores are depleted; in the absence of liver glycogen there is a rapid fall of the blood sugar to convulsive levels. As the hyperglycaemic action of intravenously infused adrenaline is probably unaffected both by hypophysectomy [in the rat—Russell & Cori, 1937] and by treatment with pituitary extracts [in the rabbit—present communication] the hypothesis of Cope & Marks is not supported. This hypothesis is also not in agreement with the observations of Himsworth & Scott [1938, 2] who found that the glycotropic substance was active in abolishing the hypoglycaemic action of insulin administered to adrenalectomized rabbits.

The injection of insulin lowers the blood sugar level of the intact animal because the production of sugar by the liver is inhibited [Cori *et al.* 1923; Cori, 1931], and because the processes of glycogen formation in the muscles and sugar oxidation in the peripheral tissues generally are stimulated [Best *et al.* 1926]. Marks [1936] found, with eviscerated cats receiving glucose intravenously, that pre-treatment with suitable anterior pituitary extracts inhibited the action of insulin in increasing the muscle glycogen level, while Himsworth & Scott [1938, 2] found that the accelerating action of insulin on the spontaneous fall of the blood sugar level which normally occurs in hepatectomized rabbits, is lacking if the rabbits are treated with a preparation of the glycotropic substance before hepatectomy. We may therefore assume that, in intact rabbits, treatment with the glycotropic substance inhibits the increase in the peripheral absorption of sugar which normally follows the administration of insulin. The complete abolition of insulin hypoglycaemia in the pituitary-treated animals shown in Fig. 2 is explicable on the basis that the action of insulin in inhibiting the production of sugar in the liver has also been abolished under the influence of the pituitary extract. The form of curve illustrated in Fig. 1 is more difficult to explain. The simplest assumption is that in the livers of these animals the glycogenolytic action of adrenaline, secreted in response to the fall of blood sugar, has been greatly exaggerated by treatment with the pituitary extract, so that the

initial fall of blood sugar causes an excessive outpouring of sugar from the liver. However, as we have seen above, there is no reason to suppose that the glycogenolytic action of adrenaline in the liver is greatly increased under the influence of our pituitary extracts. It is possible, of course, that in the liver the effectiveness of glycogenolytic nervous stimuli is increased under the influence of the glycotropic substance, and that such stimuli normally play a part in raising the blood sugar to its normal level after insulin hypoglycaemia. Definite evidence on this point is lacking, however, but it may be mentioned that Cori & Cori [1927] have found that the fall of liver glycogen which normally follows the injection of a small dose of insulin into a fasting mouse is just as marked in adrenalectomized animals as it is in normal mice. This suggests the possibility that a stimulus other than that of adrenaline secreted from the adrenal glands is acting on the liver during insulin hypoglycaemia.

Rabbits treated with pituitary extracts containing the glycotropic substance have more liver and muscle glycogen at the end of a short period of fasting than do normal animals. One would expect that the presence of a large amount of glycogen in the liver would modify the extent of the fall of blood sugar level after insulin administration, but the result illustrated in Fig. 4 shows that raising the liver glycogen by this means to over 75% above its normal fasting value had no very great influence on the hypoglycaemic response to the injection of 2 units of crystalline insulin. This suggests that the liver glycogen level cannot by itself be the controlling factor in the production of insensitivity to the hypoglycaemic action of insulin by treatment with our pituitary extracts. We must conclude, therefore, that until further results are available, the simplest assumption is that of a direct antagonism, both in the liver and peripherally, between the action of the glycotropic substance and that of insulin.

Summary

1. The glycotropic (anti-insulin) principle of the pituitary gland is not identical with any of the following pituitary substances: prolactin (i.e. the pituitary substance capable of stimulating growth of the pigeon crop gland), thyrotropic hormone, gonadotropic hormone, oxytocic and vasopressor substances.

2. Preparations of the glycotropic substance containing no detectable prolactin, and preparations of prolactin containing no detectable glycotropic substance, have been obtained.

3. In rabbits which are rendered insensitive to the hypoglycaemic action of 2 units of insulin by treatment with the glycotropic substance, the hyperglycaemic action of subcutaneously injected adrenaline is substantially increased, but the effect of intravenously infused adrenaline is not significantly affected. In such rabbits sugar tolerance is slightly diminished.

4. The glycotropic substance apparently does not antagonize the hypoglycaemic action of insulin by facilitating the glycogenolytic action in the liver of adrenaline secreted by the adrenal glands. It may directly antagonize the action of insulin both in the liver and in the peripheral tissues.

I wish to express my thanks to Dr I. W. Rowlands and Mr M. Chance who carried out the prolactin and thyrotropic hormone assays necessary for this investigation. During the initial stages of this work, at University College, I received a grant from the Medical Research Council which partly defrayed expenses, and a special grant from the Diabetic Association which provided for technical assistance. I wish to express my thanks for these grants, and also for the skilful technical assistance of Mr E. A. Woollett.

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CCI. SERUM PROTEINS IN NORMAL AND PATHOLOGICAL CONDITIONS

I. THE BLOOD SERUM OF NORMAL ANIMALS

II. HUMAN BLOOD SERUM AND PATHOLOGICAL BODY FLUIDS

III. HORSE SERUM STUDIED BY MEANS OF THE PRECIPITIN REACTION

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Although Loeb [1922] has shown that the properties of proteins need not be described vaguely as complex colloidal phenomena but that ordinary physico-chemical principles provide a basis for adequate explanation, and although chemical methods have been successfully applied in demonstrating that other biologically important substances are compounds of definite chemical constitution, nevertheless, in the case of proteins their complexity has led to the tendency to place them in a class apart. Blood serum, for example, is sometimes regarded as a single complex protein incapable of being separated into fractions of definite chemical composition, and any fractions that are obtained are regarded as artefacts [Block, 1934]. An alternative suggestion is that serum consists of innumerable fractions differing only slightly from one another or which are even interconvertible.

These views, which would render the chemical investigation of proteins of very limited value, have not been accepted in this or preceding communications but, as a working hypothesis, it has been assumed that the serum proteins consist of a limited number of chemical individuals of reproducible properties. The objects of the investigation, therefore, have been to prepare fractions as pure as possible, to seek means of identifying individual proteins and to obtain criteria of purity of each.

Although the albumin fraction of serum has been generally regarded as consisting essentially of a single protein, nevertheless as early as 1884 Halliburton, on the basis of coagulation temperatures, suggested that albumin did not consist of a single homogeneous fraction. The same conclusion was reached by Sørensen [1930] on the basis of solubility measurements and by Hewitt [1934] as the result of carbohydrate determinations and it was eventually possible [1936; 1937; 1938] to separate from the albumin fraction of serum three distinct proteins, crystalbumin, seroglycoid and globoglycoid, which are distinguishable by biological, chemical and physical methods.

Chemical and physical evidence suggests that serum globulin is a mixture of different proteins [Hewitt, 1927; 1934; 1938; Lustig & Haas, 1931; Tiselius, 1937; Stenhagen, 1938] and this communication describes attempts to carry further the identification of chemically individual proteins in the blood serum of various animals and in various body fluids of human patients.

I. THE BLOOD SERUM OF NORMAL ANIMALS

The isolation and properties of crystalbumin and seroglycoid, the main constituents of the albumin fraction, have been described previously and attention in this section is directed mainly to the globulin fractions.

In a former communication [1938] two euglobulins, designated I and II respectively, were described in the case of horse serum. Euglobulins of similar characteristics have now been obtained from human, ox and rabbit sera. Euglobulin-I separates out first when globulin fractions are dialysed in neutral solution; it is obtained in the form of a white precipitate requiring a fairly high concentration of salt to render it soluble. Euglobulin-II is precipitated when the solution from which euglobulin-I had been precipitated is either brought to *p*H 6 or else is subjected to prolonged dialysis against distilled water to remove traces of salt. Euglobulin-II is obtained in the form of a semi-liquid precipitate frequently greenish-blue in colour and it is soluble in the presence of traces of salts. In many respects it resembles the "viscous protein" of Doladilhe [1936] and its composition will be dealt with in later sections of this paper.

The water-soluble globulins or pseudoglobulins may be separated into fractions of varying properties, the fraction most readily precipitated by $(NH_4)_2SO_4$, Na_2SO_4 or NaCl having the lowest carbohydrate content [Hewitt, 1934; 1938]. The precipitin reaction of this fraction has been studied by Kendall [1937] who calls it water-soluble euglobulin or α-globulin. Since euglobulins are insoluble in water and since the latter name may be confused with α-globulin of Tiselius [1937], it is considered preferable to call the fraction *pseudoglobulin-A*.

The main bulk of the pseudoglobulin-A fraction is precipitated by one-third saturation with $(NH_4)_2SO_4$, or by saturation with NaCl, but considerable amounts remain unprecipitated and nearly all the globulin fractions so far examined contain a certain amount of this protein. It is precipitated by comparatively low concentrations (about 20 %) of alcohol in the absence of salt but contrary to common expectation higher concentrations of alcohol are necessary in order to effect precipitation in the presence of small amounts of salt.

The main pseudoglobulin fraction is not precipitable by one-third saturated $(NH_4)_2SO_4$ but is precipitated when the $(NH_4)_2SO_4$ concentration is raised to 50 % saturation. After dialysis to remove salts and euglobulins, the pseudoglobulin remaining in solution, as will be shown later, appears to consist of at least three different proteins, including pseudoglobulin-A and globoglycoid.

Globoglycoid is a globulin fraction first obtained from the albumin fraction of horse serum [Hewitt, 1938]. After removal of the main bulk of the seroglycoid the crystalbumin frequently contains considerable amounts of globoglycoid which may be separated by neutralizing the solution and precipitating with 55 % saturated $(NH_4)_2SO_4$ solution. It is now found that the serum globulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ is rich in globoglycoid, and a similar fraction was obtained by precipitation between the limits 20 and 22 % Na_2SO_4 [Hewitt, 1934]. Removal of other albumin and globulin fractions from globoglycoid is difficult and the difficulties are enhanced by the accumulation of lipins in the fraction. In the case of human, ox and rabbit serum globoglycoids the solutions are frequently milky in appearance whilst even with horse serum globoglycoid the protein is difficult to centrifuge down owing to the presence of fatty matter. As will be shown later globoglycoid is of importance from the point of view of euglobulin formation.

It is not easy to make quantitative determinations of the distribution of the various proteins in serum by fractionation processes, since the repeated reprecipitations necessary to effect purification result in large and unpredictable losses. In addition, as will be shown later, figures for euglobulin depend upon a variety of experimental conditions so that figures in the following table must be regarded as approximate only, but they serve to show the differences between the serum protein distributions of different animal species.

Table I. *Distribution of serum proteins in blood of different animals*

Results are given in g. per 100 g. total protein.

	Horse	Ox	Rabbit	Human
Euglobulin	12	17	8	17
Pseudoglobulin	46	28	16	22
Crystalbumin	33	47	66	53
Seroglycoid	9	8	10	8

In the serum of a horse immunized with diphtheria toxoid the crystalbumin content fell from 33 to 13 % of the total with a corresponding increase in the globulin fractions. Pathological changes in human serum are dealt with in a later section.

The value of the carbohydrate content in distinguishing protein fractions has been indicated in previous papers [1934; 1936; 1937; 1938]. Crystalbumin has been shown to be carbohydrate-free and seroglycoid to contain about 7 % of galactose-mannose [1936; 1937]. In the case of rabbit serum as mentioned previously [1937] the solubility relations of the constituents of the albumin fraction are such that separation is sometimes difficult as mixtures of proteins tend to be precipitated together. Some specimens of seroglycoid have a carbohydrate content appreciably greater than 7 % owing to the presence of mucoid which is readily separated by reprecipitation processes.

It is, however, the carbohydrate contents of the globulin fractions with which we are chiefly concerned at present and some of the results obtained are summarized in Table II. The modification of the Sørensen & Haugaard [1933] method previously described was used except that for colorimetric comparison, in place of the step-photometer, a photoelectric Spekker absorptiometer with filter 5 was used for some of the measurements.

Table II. *Carbohydrate contents of globulin fractions*

g. of galactose-mannose per 100 g. of protein.

Fraction	Horse	Ox	Rabbit	Human
Pseudoglobulin-A	1·4	1·4	1·5	1·5
Main pseudoglobulins	2·4	3·6	2·5	3·4
Globoglycoid	5·6	4·9	4·1	5·4
Euglobulin-I	1·8	2·1	2·6	2·8
Euglobulin-II	3·6	3·4	3·2	4·0

Just as in the cases of crystalbumin and seroglycoid the carbohydrate contents of the corresponding globulin fractions of the different animal sera resemble one another but there are several points of additional interest.

In each case pseudoglobulin-A has the lowest carbohydrate content, about 1·4 %, and preliminary experiments indicate that further purification processes, although leading to considerable losses of the material, may reduce the carbohydrate content even further. The main pseudoglobulin fractions of human and

ox serum have a higher carbohydrate content than those of horse serum probably owing to the smaller proportion present of pseudoglobulin-A with its low carbohydrate content.

Composition of euglobulin-II

Some specimens of pseudoglobulin-A and the main pseudoglobulin fraction when mixed together produce a precipitate and Kendall [1937] suggests that euglobulin is a mixture of pseudoglobulins. The matter is, however, a rather complicated one.

As mentioned above and in a previous communication [1938] there appear to be two distinct euglobulins and it is euglobulin-II of which the properties and behaviour suggest that it may be composed of mixed water-soluble pseudoglobulins. Furthermore, experiments have so far failed to produce euglobulin precipitates when horse, ox or rabbit serum protein fractions were mixed and there has been success only when human serum fractions participate in the reaction, although as will be seen later there is evidence that horse serum euglobulin-II contains pseudoglobulin-A.

The next observation was that pseudoglobulin-A gave the heaviest precipitation of euglobulin when it was mixed not with the main pseudoglobulin fraction but with globoglycoid.

Proteins from the sera of different animals were mixed and it was found possible to obtain a euglobulin-II of mixed origin, the components being derived from different animal species. The results are summarized in Table III.

Table III. *Euglobulin-II precipitates from serum globulin fractions of different animal species*

\+ indicates precipitation, 0 no precipitation.

Globoglycoid from	Pseudoglobulin-A from			
	Human	Horse	Ox	Rabbit
Human	+	0	0	0
Horse	+	0	0	0
Ox	+	0	0	0
Rabbit	+	0	0	0

It will be seen that in every case the pseudoglobulin-A constituent must be of human origin, whilst the globoglycoid may be derived from human, horse, ox or rabbit serum and yet produce a precipitate. The globoglycoid fraction is rich in lipins which have been considered to be concerned in euglobulin formation and it was decided to investigate the precipitating powers of globoglycoid freed from lipins. For the removal of lipins a more rapid modification of a method previously described [Hewitt, 1927] was used.

60 ml. of globoglycoid solution cooled to 0° were added gradually to 360 ml. of alcohol-ether (7 : 3) mixture kept at −15°. After being allowed to stand for an hour the precipitate was centrifuged down in chilled centrifuge buckets. The supernatant fluid was decanted off and the solid was suspended in 400 ml. of alcohol-ether mixture at −15°. After centrifuging the solid was washed twice in a similar manner with chilled ether which had been distilled over sodium metal. The whole process up to this stage had occupied less than 2 hr. The solid was now transferred to a Soxhlet apparatus and extracted with ether, sodium metal being present in the extraction flask to remove any water or alcohol present. After 2 hr. extraction, fresh ether and sodium were added and the extraction was continued for a further period of 3 hr. The solid was then dried

in vacuo over sulphuric acid and was finally obtained in the form of a fine white powder readily soluble in water.

It was found that this lipin-free globoglycoid still precipitated with human pseudoglobulin-A indicating that the lipin content of the globoglycoid was not an essential factor for the precipitation.

Some quantitative experiments on the amount of precipitate obtained under different experimental conditions are summarized in Table IV.

Table IV. *Amount of euglobulin precipitated by mixing human pseudoglobulin-A with various proteins*

Precipitate in mg. obtained from 10 mg. of mixed proteins.

Proportion of pseudoglobulin-A present %	Human serum pseudoglobulin (main fraction)		Serum globoglycoid from			
	*p*H 6	*p*H 7	Human	Horse	Rabbit	Ox
85	—	—	4·0	—	1·2	—
75	—	—	5·0	3·4	1·9	—
67	—	2·6	6·0	—	2·4	4·1
60	3·1	2·0	6·0	2·4	2·9	—
50	2·7	1·1	—	2·0	—	—
40	2·0	—	—	—	—	—

Precipitation is greater at *p*H 6 than at *p*H 7 as would be expected from the properties of euglobulin II already mentioned. The precipitate obtained with globoglycoid was greater than with the main pseudoglobulin fraction, suggesting that the globoglycoid present in the main pseudoglobulin fraction was the active precipitant.

Further discussion of the data described is postponed until consideration of the evidence given in later sections.

II. HUMAN BLOOD SERUM AND PATHOLOGICAL BODY FLUIDS

Human sera, urine, ascitic fluids and pleural effusions have been subjected to fractionation processes by the method already outlined with a view to detecting any considerable abnormality in the distribution of the various protein fractions.

The results of some of the fractionations are summarized in Table V. Dr Alston and Dr S. H. Robinson of the Archway Hospital Group Laboratory very kindly sent the specimens investigated.

Table V. *Protein fractions obtained from various human body fluids*

g. per 1 l. fluid.

Fraction	Normal serum	Heart failure ascitic fluid	Liver Cirrhosis ascitic fluid	Chronic parenchymatous nephritis					
				Serum	Urine	Ascitic fluid	Ascitic fluid	Pleural fluid	Pleural fluid
Pseudoglobulin-A	5·4	0·4	1·9	2·6	—	0·5	0·2	1·6	0·4
Pseudoglobulin (main fraction)	5·1	0·8	1·7	5·7	1·2	0·9	0·6	1·7	1·5
Euglobulins	8·4	1·1	1·3	5·0	1·2	1·2	0·8	2·6	1·7
Crystalbumin	25·3	8·0	3·4	3·8	1·7	1·1	1·0	3·2	2·0
Seroglycoid	3·6	2·3	2·2	5·4	1·8	0·8	0·7	1·2	0·4

The number of specimens examined is limited and it is inevitable that material is lost during fractionation processes, but despite its limitations the information obtained presents several points of interest, especially with regard to the various body fluids obtained from the case of chronic parenchymatous nephritis. All these specimens were obtained from the same patient, a man aged 51 with albuminuria and generalized oedema of a recurrent type; the blood urea concentration varied from 0·06 to 0·11 %.

The low albumin : globulin ratio in such cases of nephritis is well-known but we now know that the albumin fraction includes seroglycoid and globoglycoid in addition to crystalbumin. It is interesting, therefore, to consider the crystalbumin concentration apart from the other components of the albumin fraction. Thus the albumin : globulin ratios of the normal and nephritic sera in the table were 1·4 and 0·8 respectively, whilst the ratios of crystalbumin : "other proteins" were 0·8 and 0·2 respectively. The difference is, therefore, much more striking when the crystalbumin alone is considered instead of the whole albumin fraction. The explanation of this is discussed later.

There is some evidence of a decreased proportion of pseudoglobulin-A in the globulin fraction of the nephritic specimens as has been indicated by the precipitin tests of Kendall [1937].

The properties so far examined of the various fractions were similar irrespective of whether their origin was serum, urine, ascitic fluid or pleural effusion. The carbohydrate content of the pseudoglobulin-A fractions approximated to 1·5 % and that of the globoglycoids to 4 or 5 % although the quantity of material available precluded careful purification. The carbohydrate contents of the main pseudoglobulin fractions varied from 2·3 to 4·4 % according to the proportions present of the various proteins comprising the fraction. Pseudoglobulin-A and globoglycoid derived from different body fluids, when mixed, yielded a precipitate of euglobulin-II just as in the case of proteins obtained from serum.

The high carbohydrate content (16·4 %) of a crude specimen of seroglycoid from nephritic ascitic fluid suggested the presence of the mucoid investigated by Zanetti [1897] and Bywaters [1909], and fractionation with $(NH_4)_2SO_4$ resulted in the separation of two fractions, one mainly seroglycoid containing 8·5 % of galactose-mannose and the other, probably mucoid containing 20 % of carbohydrate.

III. HORSE SERUM STUDIED BY MEANS OF THE PRECIPITIN REACTION

The difficulty of obtaining pure antigens and the corresponding specific antisera and the ease with which biological tests are influenced by slight variations in experimental conditions, must necessarily set limits to the value of the precipitin reaction, and the accuracy attainable in chemical analysis is not reached. Nevertheless, precipitin tests are of great value for the qualitative examination of the homogeneity of protein fractions. Results of some quantitative significance may be obtained by observation of the first tube in a graded series of dilutions to exhibit flocculation [Dean & Webb, 1926], and, in conjunction with this method, by determining the nitrogen content of the floccules precipitated under appropriate conditions [Heidelberger & Kendall, 1932].

The importance of the route and mode of inoculation in the preparation of antisera is apparently greater than is generally appreciated and this will be discussed in a later section.

The antisera used were prepared by twice-weekly intravenous injections into rabbits of suspensions of alum-precipitated proteins over a period of 3 or 4 weeks, the course of injections being repeated if necessary. Antisera to four different fractions were investigated and they are described below.

Antipseudoglobulin-A serum reacted only with pseudoglobulin-A and on other zone of precipitation was observed.

Antigloboglycoid serum reacted principally with globoglycoid but a subsidiary zone of precipitation was observed and this was traced to the presence of antibody to crystalbumin, which was removed by absorption in the optimal proportion with pure crystalbumin.

Anticrystalbumin and *antiseroglycoid sera* reacted only with the homologous antigens.

Titrations of the antigens with the homologous antisera are summarized in Table VI.

Table VI. *Precipitin reactions of antigens with their homologous antisera*

Antisera were diluted 1 in 20 and mixed with equal volumes of antigen of dilutions given in table.

Antigen	100	300	1000	3000	10,000	30,000	100,000
Pseudoglobulin-A	–	–	–	–	+ + +	+ +	+
Globoglycoid	–	+	+ + +	+ +	+	–	–
Crystalbumin	–	–	–	–	+ + +	+ +	+
Seroglycoid	–	–	–	–	+ + +	+ +	+

Having thus obtained four specific sera each reacting with one of the protein constituents of serum, it became possible to investigate the composition of various sera and serum protein fractions. The method of carrying out the precipitin tests was to mix equal volumes of antiserum, diluted 1 in 20, with graded dilutions of the protein solution ranging from 1 % down to one-hundred thousandth or less of this concentration, and to observe the tubes at intervals for the first appearance of opalescence and flocculation.

Tested in this way good specimens of pseudoglobulin-A and crystalbumin behaved as homogeneous proteins, seroglycoid contained small amounts of crystalbumin whilst globoglycoid was found to contain small amounts of pseudoglobulin-A and crystalbumin. The main pseudoglobulin fraction also contained pseudoglobulin-A and it appears that nearly all globulin fractions contain this protein in greater or less amount.

Suitable proportions were found by preliminary tests in graded dilutions, and quantitative determinations were then carried out [Heidelberger & Kendall, 1932] by mixing the antisera with various fractions, keeping the proportions present in the region of antibody excess. After standing overnight in the ice-chest the precipitates were centrifuged down, washed with saline and the nitrogen contents determined by the micro-Kjeldahl method, the protein : nitrogen factor of 6·45 being used throughout in the calculations.

The antisera used were first standardized by determining the amount of precipitate produced by known amounts of the appropriate protein and plotting the curve connecting these quantities (Fig. 1). The amount of any of the four

proteins present in a fraction may be ascertained by determining the amount of precipitate obtained with the appropriate antiserum and reading off from the curve the corresponding quantity of antigen.

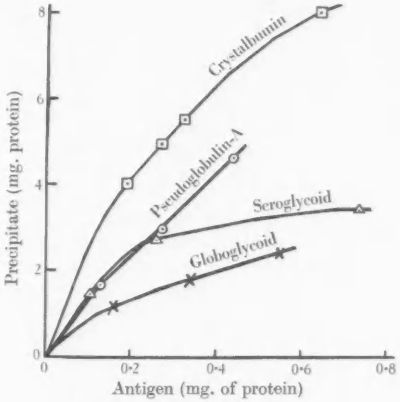

Fig. 1. Amount of protein precipitated on adding various horse serum proteins to the corresponding rabbit antisera.

The results are summarized in Tables VII, VIII, IX and X.

Table VII. *Pseudoglobulin-A antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Pseudoglobulin-A present (calculated) mg.
Pseudoglobulin-A	0·13 mg.	1·68	—
,,	0·27 mg.	2·99	—
,,	0·44 mg.	4·61	—
Horse serum 471	0·02 ml.	5·02	0·490
489	0·02 ml.	5·68	0·565
478	0·0033 ml.	3·37	0·310
Globoglycoid	2·2 mg.	2·95	0·270
Total globulins	0·91 mg.	5·85	0·585
Euglobulin-II	0·45 mg.	3·08	0·283
Pseudoglobulin (main fraction)	0·41 mg.	3·16	0·290

Table VIII. *Globoglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Globoglycoid present (calculated) mg.
Globoglycoid	0·16 mg.	1·17	—
,,	0·34 mg.	1·79	—
,,	0·55 mg.	2·39	—
Horse serum 471	0·10 ml.	2·07	0·46
489	0·10 ml.	2·08	0·47
478	0·10 ml.	2·36	0·59
Pseudoglobulin (main fraction)	3·50 mg.	1·68	0·34

Table IX. *Crystalbumin antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Crystalbumin present (calculated) mg.
Crystalbumin	0·19 mg.	4·02	—
,,	0·27 mg.	4·96	—
,,	0·32 mg.	5·52	—
,,	0·64 mg.	8·05	—
Horse serum 471	0·02 ml.	5·78	0·345
489	0·02 ml.	6·30	0·400
478	0·07 ml.	8·05	0·640
Globoglycoid	4·40 mg.	5·24	0·290

Table X. *Seroglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Seroglycoid present (calculated) mg.
Seroglycoid	0·11 mg.	1·45	—
,,	0·26 mg.	2·71	—
,,	0·74 mg.	3·43	—
Horse serum 471	0·10 ml.	3·03	0·35
489	0·02 ml.	1·45	0·11
478	0·10 ml.	2·71	0·26

Some interesting conclusions can be drawn from the tables. The main pseudoglobulin fraction of the horse serum examined was found to consist of 70 % of pseudoglobulin-A and 10 % of globoglycoid, leaving 20 % to be accounted for in the form of some other pseudoglobulin. Human serum pseudoglobulin contains only 30 % of pseudoglobulin-A according to Kendall [1937]. The higher proportion of pseudoglobulin-A in horse serum pseudoglobulin probably accounts for the lower carbohydrate content of horse serum fractions compared with human serum fractions, as mentioned previously.

Globoglycoid was first separated from the albumin fraction [Hewitt, 1938], but precipitin tests now show that the main pseudoglobulin fraction also contains about 10 % of globoglycoid. This is supported by the fact that the pseudoglobulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ has the properties of globoglycoid.

Euglobulin-II from horse serum was found to contain 63 % of pseudoglobulin-A; this may be compared with the figures 48–65 % obtained for human euglobulin [Kendall, 1937].

The difficulties of purifying globoglycoid have already been discussed and they are illustrated by precipitin reaction results which indicated that the specimen of globoglycoid examined contained 6 % of crystalbumin and 12 % of pseudoglobulin-A.

Results of the precipitin analysis of whole horse serum are of particular interest and are summarized in Table XI.

Table XI. *Analysis of horse serum by precipitin reactions*

Results are given in g. of protein per 100 ml. of serum.

Fraction	Normal horse No. 471	Normal horse No. 489	Diphtheria antitoxin horse No. 478
Pseudoglobulin-A	2·45	2·83	9·30
Globoglycoid	0·46	0·47	0·59
Crystalbumin	1·73	2·00	0·91
Seroglycoid	0·35	0·55	0·26
Other proteins	2·02	1·82	1·34

The diphtheria antitoxin horse (No. 478) had received a series of injections of formalized diphtheria toxin, the antitoxin content of the serum had risen to 500 A.U. per 1 ml. and was investigated because the protein content had increased considerably. It will be seen that the pseudoglobulin-A content had increased threefold, whilst the crystalbumin content had fallen to less than half the normal value. It is possible that the fall in crystalbumin content might be a compensatory mechanism to balance the increased osmotic pressure of the serum caused by the increase in globulin concentration.

. The figures given for "other proteins" present in serum are obtained by difference and are approximate only. These other proteins probably include euglobulin-I, and a pseudoglobulin. This pseudoglobulin is difficult to isolate owing to the difficulties of removing pseudoglobulin-A, etc.

The antisera were used to study the absorption of proteins from the peritoneal cavity. A rabbit was injected intraperitoneally with 10 ml. of horse serum. At intervals blood was withdrawn from the ear vein and the amounts of the various horse serum proteins in the rabbit's blood serum were followed by carrying out precipitin tests, using the optimal proportions method [Dean & Webb, 1926]; the considerable quantities of antisera required for the Heidelberger & Kendall [1932] method were not available. By comparison of these results with those obtained using purified antigens of known protein content it was possible to calculate the approximate quantity of each protein present.

Table XII. *Amount of horse serum proteins present in the blood serum of a rabbit injected intraperitoneally with 10 ml. of horse serum*

mg. protein per 100 ml. rabbit serum.

Time hours	Pseudoglobulin-A	Globoglycoid	Crystalbumin	Seroglycoid
0·25	25	10	20	6
1	80	—	140	—
4	100	100	210	60
24	80	—	180	—
96	80	—	140	—
336	25	—	7	—

The highest concentration of the foreign proteins was reached after 1–4 hr. and diminished quite slowly.

The serum and cerebrospinal fluid of several patients who had received intravenous injections of horse serum containing meningococcus antitoxin were also examined by the optimal proportions technique. Dr Burtenshaw of the Southern Group Laboratory kindly sent these specimens. The horse serum proteins were readily detectable in the presence of human serum proteins, and the highest concentrations recorded were: in the serum, 800 mg. of pseudoglobulin-A and 150 mg. of crystalbumin; and in the cerebrospinal fluid, 9 mg. of pseudoglobulin-A and 4 mg. of crystalbumin. The results refer to the quantity present in 100 ml.

Discussion

It is becoming increasingly clear that serum cannot be regarded as a mixture of only two proteins, globulin and albumin. The albumin fraction has been separated into three different proteins, crystalbumin, seroglycoid, and globoglycoid, of distinctive properties [Hewitt, 1934; 1936; 1937; 1938]. Dr Philpot kindly examined a specimen of seroglycoid in the ultracentrifuge and reported that the sedimentation constant was not appreciably different from that of crystalbumin. Albumin prepared by electrophoresis [Tiselius, 1937] still contains

large amounts of seroglycoid as shown by its high carbohydrate content. Thus although both by chemical and precipitin tests crystalbumin and seroglycoid have been shown to be distinct and different proteins, their properties are such that certain physical methods may fail to differentiate them. Kekwick [1938] obtained an albumin fraction which by ultracentrifugal and electrophoretic methods appeared to be homogeneous, yet which by precipitin tests [Gell & Yuill, 1938] was found to be a mixture.

Previous work has revealed the presence in serum of globulins of different properties [Hewitt, 1927; 1934; 1938]. Pseudoglobulins may be obtained by fractionation with $(NH_4)_2SO_4$, Na_2SO_4 or NaCl, with carbohydrate contents ranging from 1·4 to 6·0 % (calculated as galactose-mannose). The pseudoglobulin of lowest carbohydrate content, about 1·4 %, is obtained by several reprecipitations with one-third saturated $(NH_4)_2SO_4$ solution. As the result of a study of precipitin reactions of a similar fraction obtained from human serum Kendall [1937] concluded that it was a single protein. The alternative names suggested for this protein both have disadvantanges and without making any assumption as to its identity the protein is therefore described as *pseudoglobulin-A*. Closely similar pseudoglobulin-A fractions have been obtained from human, horse, ox and rabbit sera. There is, however, a difference between pseudoglobulin-A obtained from human sera and that from the sera of other animals, and this difference will be described in the next few paragraphs.

Under certain conditions, when pseudoglobulin-A and the main pseudoglobulin fraction are mixed a precipitate is obtained [Kendall, 1937]. The semi-fluid consistency of this precipitate resembles that of euglobulin-II, and precipitin tests also indicate that pseudoglobulin-A is a constituent of euglobulin-II. It has been found that pseudoglobulin-A gives a heavier precipitate of euglobulin with globoglycoid than with the main pseudoglobulin fraction. This, and the fact that the main pseudoglobulin fraction has been found to contain globoglycoid makes it seem likely that this latter protein is the active precipitant. Since much lipin accumulates in the globoglycoid fraction it was thought possible that this was an essential factor but it was found that globoglycoid freed from lipin still acted as a precipitant.

Mixing pseudoglobulin-A and globoglycoid from human sera (or other body fluids) resulted in precipitation of euglobulin but this did not occur when the appropriate fractions of the sera of other animal species were mixed. Furthermore, mixture of human serum globoglycoid with pseudoglobulin-A from horse, ox and rabbit sera gave no precipitate. On the other hand pseudoglobulin-A from human serum gave a precipitate when it was mixed with human, horse, ox or rabbit serum globoglycoid. It is of some interest that it is possible to prepare a euglobulin of mixed origin derived from two different animal species.

Antisera have been prepared in rabbits reacting specifically with pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid from horse serum. With these antisera it has been possible, using a quantitative precipitin technique, to obtain analytical figures for the composition of various fractions and for horse serum itself. It is now possible to quote figures for the bulk of the serum protein in terms, not of arbitrary fractions, but of proteins which although difficult to purify nevertheless may be regarded as chemical entities.

The tentative figures obtained for normal horse serum were: pseudoglobulin-A, 2·6 %; globoglycoid, 0·5 %; crystalbumin, 1·9 %; seroglycoid, 0·5 %; other proteins, 1·9 %. The figure for "other proteins" is arrived at by difference and is not accurately determined but it probably includes a new pseudoglobulin fraction and euglobulin-I as well as traces of mucoid.

Precipitin analysis of the serum of a horse after a series of injections of formalized diphtheria toxin showed that the pseudoglobulin-A fraction had increased threefold to 9·3 % whilst the crystalbumin content had fallen to 0·9 %, less than half the normal value. The reason for the increase in pseudoglobulin-A is obscure since diphtheria antitoxin appears not to be associated with this fraction [Hewitt, 1934; 1938].

Fractionation of the proteins present in human serum and pathological body fluids revealed that, as in the case of urinary and serum albumin [1927; 1929], the proteins from different sources could not be distinguished from each other. Thus pseudoglobulin-A and globoglycoid when mixed gave a precipitate of euglobulin regardless of whether the proteins originated in serum, urine, ascitic fluid or pleural effusions. The carbohydrate contents of the proteins from different sources also were similar.

It was found that there was a very marked fall in the crystalbumin content of the serum of a case of chronic parenchymatous nephritis but the seroglycoid constituent of the albumin fraction was not diminished in amount. Seroglycoid was present in considerable amounts in nephritic urine, ascitic fluid and pleural effusions but yet the concentration in the serum had not fallen as it had in the case of crystalbumin. It would seem, therefore, that mechanical loss through the kidney did not of itself account for the diminished amount of crystalbumin in the blood stream, and it appears that regeneration of crystalbumin must proceed more slowly than that of seroglycoid or the globulins. Perhaps it is significant in this connexion that crystalbumin, alone among the serum proteins so far isolated, is chemically different in not containing polysaccharide in the molecule. The slow regeneration of albumin compared with globulins after plasmaphoresis was observed by Kerr *et al.* [1918]. Liver damage appears to delay the resynthesis of albumin [Foley *et al.* 1937]. As mentioned previously the explanation is lacking of the large increase in pseudoglobulin-A content of the serum of horses injected with diphtheria toxin and indeed the whole problem of serum synthesis is obscure.

The species specificity of the various serum proteins made it possible to study the appearance of foreign proteins in the blood stream or cerebrospinal fluid after intraperitoneal or intravenous injection of foreign protein. A rabbit received an intraperitoneal injection of horse serum. Within a quarter of an hour after the injection horse serum pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid could be detected in the rabbit's blood by means of precipitin tests. Disappearance of the foreign proteins from the rabbit's blood was slow, and traces were still detectable after a fortnight. On the other hand, it is reported that after intravenous injection of egg albumin the foreign protein disappeared from the blood stream very rapidly, 94 % having gone after 2 hr. and 99·6 % after 24 hr. [Kenton, 1938].

After intravenous injection of horse serum containing meningococcus antitoxin into patients, traces of pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid.

The question of the relative antigenicities of different proteins is complicated; there is, for example, considerable divergence in the literature concerning the antigenicity of serum albumin. That albumin is non-antigenic is maintained by some workers [Nolf, 1900; Landsteiner & Calvo, 1902; Ruppel *et al.* 1923] whilst others find it yields a specific antibody [Michaelis, 1902; Hektoen & Welker, 1924; Kabat & Heidelberger, 1937; Gell & Yuill, 1938]. The confusion was in some measure due to the fact that the serum albumin fraction contains at least three different antigenic proteins, crystalbumin, seroglycoid and globoglycoid,

each giving rise to a separate antibody, but there appears to be an additional source of confusion. It was reported previously that purified crystalbumin was only feebly antigenic when injected intraperitoneally even in large doses, whilst serum globulins, under these conditions are potent antigens [1937]. When injected intravenously crystalbumin is antigenic [Kabat & Heidelberger, 1937; Gell & Yuill, 1938] especially when the injected material is in the form of an alum suspension. The use of alum in enhancing antigenic activity was reported by Glenny *et al.* [1926]. Not only does the antigenic activity increase in the presence of alum but the risks of anaphylaxis after intravenous injection, previously reported [Hewitt, 1937], appear to be minimized. It is important, therefore, in attempting to evaluate the relative antigenicities of different proteins to take into account the route and method of injecting the antigens.

Summary

1. Attempts have been continued to separate serum into chemically distinct homogeneous individual proteins.

2. Pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid have been detected in human, horse, ox and rabbit sera.

3. Conditions have been studied under which mixing of pseudoglobulin-A and globoglycoid produces a precipitate of euglobulin-II.

4. In a nephritic patient the protein fractions were similar whether derived from serum, urine, ascitic fluid or pleural effusion and they could not be distinguished from normal serum proteins.

5. Both seroglycoid and crystalbumin were excreted in the urine of a nephritic patient in considerable amounts and the crystalbumin content of the serum fell to a low value, but the seroglycoid content did not fall appreciably. A different mechanism of regeneration of different serum proteins is indicated.

6. Specific precipitating sera were prepared for the four horse serum proteins, pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid. The route and method of injection of crystalbumin have marked effects on the antigenicity.

7. Approximate figures for the composition of normal horse serum in terms of four of the individual protein constituents have been obtained by means of quantitative precipitin tests. The results are: pseudoglobulin-A 2·6%; globoglycoid 0·5%; crystalbumin 1·9%; seroglycoid 0·5%; other proteins 1·9%.

8. In the serum of a horse immunized against diphtheria toxin the pseudoglobulin-A content had increased threefold and the crystalbumin content had fallen to half its normal value.

9. A rabbit was injected intraperitoneally with horse serum and the various horse serum protein fractions were detected by precipitin tests in the rabbit's blood stream within 15 min. of the injection. The maximum concentration of foreign protein was reached after 1 to 4 hr. and fell only slowly, traces being still detectable after a fortnight's interval.

10. Traces of horse serum pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid of patients injected intravenously with horse serum.

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CCII. THE POLYSACCHARIDE CONTENT AND REDUCING POWER OF PROTEINS AND OF THEIR DIGEST PRODUCTS

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THE carbohydrate contents of proteins are of value in differentiating individual serum proteins and in following the course of fractionation processes [Hewitt, 1934–1938]. Apart from these practical considerations, however, the presence of carbohydrate groups or polysaccharides in proteins is of theoretical interest also, from the point of view of the structure of the protein molecule.

In order to separate the polysaccharide completely from protein material very drastic chemical treatment is necessary [Levene & Mori, 1929; Rimington, 1931; Bierry, 1934; Onoe, 1936]. Whilst this may be taken as evidence that the polysaccharide forms an integral part of the protein molecule, it also raises doubts whether the constitution of the polysaccharide present in the protein molecule is necessarily the same as that of the isolated material, particularly when the yield of purified polysaccharide is very low. Any data obtainable without previous isolation of the polysaccharide are therefore of interest, so it was decided to attempt to correlate the reducing powers of various proteins with their polysaccharide contents. Below are presented data on the carbohydrate contents, glucosamine contents and reducing powers of various proteins, and of the effect of various hydrolytic and enzymic digestion processes.

EXPERIMENTAL

Carbohydrate determinations. A modification of the orcinol method [Tillmans & Philippi, 1929; Sørensen & Haugaard, 1933] was used as previously described, colorimetric comparison being made either in a Stufenphotometer, or photoelectrically using a green filter. For serum proteins results are quoted in terms of galactose-mannose and for egg proteins in terms of mannose.

Reducing powers. The reducing powers of solutions were determined by the Hagedorn & Jensen [1923] method as modified by Hanes [1929] and Hulme & Narain [1931] except that deproteinization with $ZnSO_4$ and NaOH was omitted, the protein solution being heated with the ferricyanide reagent.

Glucosamine determinations. The colorimetric methods described by Zuckerkandl & Messiner-Klebermass [1931], Elson & Morgan [1933], Nilsson [1936], and Palmer *et al.* [1937] were modified as follows for the determination of the glucosamine content of proteins. Hydrolysis was effected by heating the protein solution in a boiling water bath for 4 hr. with 2*N* HCl, and the solution was cooled and neutralized with NaOH. The temporary existence, during the neutralization, of a slightly alkaline reaction was not found deleterious, although the importance of avoiding alkalinity has been stressed by some workers. After filtration through filter paper to remove humin 1 ml. of the solution, diluted to contain 0·05–0·3 mg. glucosamine was heated with 1 ml. acetylacetone solution (2 % in 0·5*N* Na_2CO_3) in a boiling water bath for 15 min. Irregular results were obtained until a simple but effective form of reflux condensation was resorted to. The solutions were contained in $6 \times 0{\cdot}75$ in. test tubes drawn out into a

constriction at the upper end and resting in this narrow neck was placed a $3 \times 0{\cdot}4$ in. test tube filled with cold water. After heating, the tubes were cooled by immersion in cold water and 5 ml. of alcohol were added. The ethyl alcohol had been purified by heating with Ag_2O and NaOH and redistilled. 1 ml. of Ehrlich's reagent was added and the colour was allowed to develop for 30 min. Ehrlich's reagent was prepared by dissolving 0·8 g. purified dimethylaminobenzaldehyde in 30 ml. purified ethyl alcohol and adding 30 ml. concentrated HCl. The *p*-dimethylaminobenzaldehyde was purified by dissolving in concentrated HCl, diluting with water and adding sodium acetate solution. The first yellowish precipitates obtained were rejected, and further addition of sodium acetate produced a white precipitate, which yielded a satisfactory reagent. Colorimetric comparisons were carried out in a Stufenphotometer using 1 cm. cells and an S 53 filter, glucosamine solutions being examined at the same time.

Protein preparations. The blood serum proteins used were prepared as described previously. The casein, edestin, gelatin and amino-acids investigated were commercial specimens. The ovalbumin specimens used were repeatedly crystallized and the ovomucoid was obtained from the mother liquors after the successive removal of globulins, albumin and conalbumin. The ovalbumin was quite colourless but the ovomucoid was lemon yellow in colour and when pure contained no heat-coagulable protein.

Results. In Table I are summarized mean values for the glucosamine and carbohydrate contents of various proteins. The seroglycoid and globoglycoid preparations contained some crystalbumin and had correspondingly decreased carbohydrate contents.

Table I. *Galactose-mannose and glucosamine contents of proteins of blood serum and egg white*

(g. per 100 g. protein)

Protein	Galactose-mannose (G.-M.)	Glucosamine (Ga.)	Ratio $\left(\frac{\text{G.-M.}}{\text{Ga.}}\right)$
Seroglycoid	5·6	2·7	2·1
Crystalbumin	<0·05	<0·05	—
Globoglycoid	6·2	2·9	2·1
Pseudoglobulin	2·2	1·1	2·0
Euglobulin-II	2·7	0·8	3·5
Ovalbumin	1·8*	0·8	2·3
Ovomucoid	10·5*	9·5	1·1

* In the case of egg white proteins results are quoted in terms of mannose.

It has been shown in previous communications that purified crystalbumin contains no carbohydrate and in the present experiments it was found, as anticipated, that no glucosamine could be detected in crystalbumin hydrolysates. In the case of the other serum proteins in which the polysaccharide present is generally considered to be galactose-mannose-glucosamine the ratio of galactose-mannose content to glucosamine content should be 2 : 1. With seroglycoid, globoglycoid and pseudoglobulin the deviation from this ratio does not exceed the probable experimental error; in the case of euglobulin-II the ratio appears to be high but the reason for this is not at present apparent.

In the case of ovalbumin the mannose : glucosamine ratio is in agreement with the presence of dimannose-glucosamine but with ovomucoid there are equimolecular amounts of mannose and glucosamine suggesting that the polysaccharide present is built up of mannose-glucosamine units. This will be commented upon later.

In Table II are summarized the reducing powers of various protein substances, using the Hagedorn-Jensen method; the figures quoted are in terms of glucose. The polysaccharide contents are calculated in terms of galactose-mannose-glucosamine for the serum proteins, dimannose-glucosamine for ovalbumin and mannose-glucosamine for ovomucoid.

Table II. *Polysaccharide contents and reducing powers of various proteins, etc.*

(g. carbohydrate per 100 g. protein)

Protein	Polysaccharide content	Reducing power (glucose)
Euglobulin-II	4·1	11·9
Pseudoglobulin	3·3	12·5
Globoglycoid	9·3	13·5
Crystalbumin	>0·05	17·0
Seroglycoid	8·4	14·4
Ovalbumin	2·6	9·5
Ovomucoid	20·0	23·0
Casein	0·5	7·5
Edestin	>0·1	7·5
Gelatin	0·5	0·9
Cystine	0	15·0
Glutamic acid	0	0·1
Glycine	0	0
Tryptophan	0	80·0
Tyrosine	0	100·0

It will be seen at once from Table II that the reducing power of the proteins is not dependent upon the polysaccharide content. Of the serum proteins crystalbumin has the highest reducing power although it contains no carbohydrate.

Carbohydrate, when present, probably contributes to the reducing power but it is necessary to seek some other constituent of the protein molecule, to account for a considerable portion, or in some cases all, of the reducing power. Glycine and glutamic acid were found to have no reducing power, cystine had about one-seventh of the reducing power of glucose, tyrosine had approximately the same reducing activity as dextrose and tryptophan four-fifths of the reducing power.

It is significant that gelatin has an extremely low reducing activity since it is the only protein investigated containing negligibly small amounts of cystine, tryptophan and tyrosine in addition to having a low carbohydrate content.

The Hagedorn-Jensen method for the determination of reducing power includes the liberation of free iodine at one stage and it was thought possible

Table III. *Comparison of the apparent reducing powers of proteins, etc., at room temperature and when heated with ferricyanide*

Protein	Apparent reducing power	
	When heated	At room temperature
Crystalbumin	17·0	0·5
Pseudoglobulin	12·5	0·4
Globoglycoid	13·5	1·1
Seroglycoid	14·4	0·9
Casein	7·5	1·5
Edestin	7·5	1·5
Cystine	15·0	1·0
Tryptophan	80·0	60·0
Tyrosine	100·0	45·0

that iodination of the protein might account for a substantial portion of the reducing power. In the next series of experiments, therefore, heating of the protein with the ferricyanide was omitted and the protein was mixed with the reagents in the cold and titration was conducted immediately. The figures obtained are summarized in Table III.

In the case of tryptophan, and to a less extent with tyrosine "cold" reducing power, probably associated with iodination, is very marked, but in the case of the serum proteins from 92 to 97 % of the Hagedorn-Jensen reducing power is observed only when the protein is heated with ferricyanide.

Effects of acid hydrolysis

Heating with 2*N* HCl for 4 hr. was sufficient to liberate the glucosamine present in the protein, but this heating had no appreciable effect on the galactose-mannose content of the protein as determined by the usual orcinol method. In most cases the effect of the heating with acid on the reducing power was also negligible. The exceptions were ovomucoid, in which the reducing power increased from 23 to 27 % (calculated as dextrose), and crystalbumin, in which the reducing power fell from 17 to 8 % during the heating with acid.

Enzymic hydrolysis

Solutions of pseudoglobulin, seroglycoid, globoglycoid and ovalbumin containing 0·125*N* HCl and 0·4 % pepsin were incubated at 52° for 18 hr. Acetone (from 4 to 9 vol.) was added to the cooled digestion mixture and the precipitate was centrifuged off, washed with acetone and ether and dried *in vacuo*. The polysaccharide contents of the dried products were 19, 19, 24 and 18 % respectively—values from three to six times higher than those of the original proteins. Products containing even higher carbohydrate contents may readily be obtained by modification of the conditions, as the following example illustrates. A globoglycoid solution of *p*H 1·03 containing 0·4 % pepsin was incubated at 52° for 18 hr. The digest product precipitated by 80 % acetone contained 35 % of polysaccharide (calculated as galactose-mannose-glucosamine).

The effect of experimental conditions on the digestion of pseudoglobulin containing diphtheria antitoxin is seen in Table IV. In each case 5 ml. of 3·5 % pseudoglobulin were treated with varying amounts of HCl and pepsin and the mixture was incubated for 2 hr. at 52°. After neutralizing the digestion mixture the flocculation titre was determined under standardized conditions.

Table IV. *Pepsin digestion of diphtheria antitoxin under different experimental conditions*

Digest no.	0·1 *N* HCl ml.	0·1 % pepsin ml.	Flocculation Titre A.U./1 ml.	Flocculation Time min.
1	2·0	0·5	0	—
2	1·1	0·5	250	14
3	0·9	0·5	350	14
4	0·9	0·1	350	80
5	0·7	0·5	450	22
6	0·5	0·5	500	45
7	0·5	2·0	550	14
8	0	0	600	38

It will be seen that the destruction of antitoxin appears to be due to acid and that the presence of pepsin has some protective effect as claimed by Parfentjev [1936]. The digest products in some cases actually flocculate more rapidly than the original product although chemical manipulation usually lengthens the flocculation time. The accelerated flocculation of digested material was noted by Pope & Healey [1938]. An attempt was made to purify digest product No. 3 by first removing the material precipitated by addition of 0·5 vol. acetone and then collecting and drying the product precipitated on addition of a further 1·5 vol. of acetone. When dissolved in water the flocculation time was still only 17 min. This indicates a much greater degree of stability than that of undigested antitoxin. Purification of digest No. 7 by this method was less successful, the flocculation time increasing to 43 min. The carbohydrate content of an experimental batch of digest antitoxin kindly supplied by Mr Pope was 2·5 %, a figure falling within the limits observed for normal horse serum pseudoglobulin.

Discussion

It has been suggested that ovalbumin contains no polysaccharide and that the carbohydrate detected in specimens of this protein is due to the presence of ovomucoid [Levene & Mori, 1929]. The figures given by Levene & Mori for the polysaccharide content of these proteins were obtained by actual isolation, a process which would be expected to give a low yield. The results reported, 0·26 % for ovalbumin and 5·1 % for ovomucoid are, in fact, much lower than the figures, 2·7 and 21 % respectively, obtained in the present investigation. Figures of the same order as the present values have been reported by Sørensen [1934] and Onoe [1936]. Repeated crystallization has failed to reduce the carbohydrate content of ovalbumin.

In ovalbumin the ratio of mannose to glucosamine approximates to 2 : 1 and in ovomucoid it is close to 1 : 1. It would seem, therefore, that the polysaccharides present in the two proteins are different, in one case being composed of dimannose-glucosamine and in the other of mannose-glucosamine units. In the present work no attempt has been made to distinguish between glucosamine and its acetyl derivative. The latter is said to be present in ovomucoid by Onoe [1936]. There is thus considerable weight of evidence that ovalbumin contains polysaccharide in the molecule, unlike serum crystalbumin: the carbohydrate content of serum albumin has been traced to the presence of seroglycoid, globoglycoid, etc. [Hewitt, 1934; 1937; 1938].

It was thought possible that determination of the reducing power of proteins might be of value in gauging the amount and nature of the polysaccharides present in the protein molecule and in following the liberation of polysaccharides during the hydrolysis of proteins. The Hagedorn-Jensen method has been used for this purpose. It has been found, however, that the reducing power of proteins bears little relation to the carbohydrate content. Crystalbumin, for example, which contains no polysaccharide has the highest reducing power of all the serum proteins investigated. With each protein the reducing power was greater than could be accounted for by the carbohydrate content even assuming that the whole of the carbohydrate was free to exert its whole reducing effect.

Various amino-acids were examined and it was found that although glycine and glutamic acid did not reduce the reagents used, other amino-acids had very pronounced reducing effects. Cystine had 15 % of the reducing power of dextrose and tryptophan 80 %, whilst tyrosine had about the same reducing activity as

an equal weight of dextrose. That the reducing power of proteins is due in part to amino-acids such as cystine, tyrosine and tryptophan, receives support from the behaviour of gelatin. Gelatin was the only protein material investigated which is deficient in these particular amino-acids as well as in carbohydrate and it was the only protein with a negligibly small reducing activity. Four hours' heating with 2*N* HCl is sufficient to liberate glucosamine from serum and egg white proteins, but the reducing power is not increased by this treatment except in the case of ovomucoid where there was an increase from 23 % (calculated as glucose) before heating to 27 % after hydrolysis. Meyer *et al.* [1936] have attempted to show by measurements of reducing power that the bacteriolytic activity of lysozyme is due to splitting off polysaccharides from glycoproteins, but it would seem from the present experiments that the technical difficulties in following the liberation of carbohydrates from proteins by reducing power determinations are very great. In the case of crystalbumin the effect of acid hydrolysis was an unexplained fall in the reducing power to about half its original value.

By digesting serum and egg proteins with pepsin and precipitation with acetone, peptones may readily be prepared with high carbohydrate contents, the highest carbohydrate content so far observed being 35 %. Such carbohydrate-rich peptones will receive further study. In a recent short note Ogston [1938], using a commercial enzyme preparation, split off rather less than half the carbohydrate from a specimen of pseudoglobulin, leaving one-third of the protein with an unchanged ultracentrifugal sedimentation constant. Full experimental details are lacking but it is possible that one of the constituents of the pseudoglobulin fraction [Tiselius, 1937; Hewitt, 1934; 1938] may be preferentially hydrolysed.

Summary

1. The glucosamine contents of pseudoglobulin, globoglycoid and seroglycoid are in rough agreement with the generally accepted view that the polysaccharide present in the proteins is composed of units having the composition galactose-mannose-glucosamine. Crystalbumin contains no glucosamine.

2. The glucosamine contents of the egg white proteins are in agreement with the view that ovomucoid contains mannose-glucosamine and that ovalbumin contains dimannose-glucosamine.

3. Evidence is presented disproving the view that the polysaccharide present in ovalbumin is due to the presence of ovomucoid.

4. The reducing power of proteins has been traced to the presence of various amino-acids such as tyrosine, tryptophan and cystine as well as to carbohydrates.

5. The reducing power of many proteins is not increased after acid hydrolysis and the course of hydrolysis cannot therefore be followed by reducing power determinations.

6. Carbohydrate-rich peptones may readily be prepared from proteins by peptic digestion followed by acetone precipitation.

7. A few observations have been made on the effect of peptic digestion on diphtheria antitoxin globulins.

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CCIII. THE PHOTOCHEMICAL DECOMPOSITION OF *l*-ASCORBIC ACID

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During the last few years evidence has been accumulating to show that the oxidation of *l*-ascorbic acid is catalysed by visible light in the presence but not in the absence of certain sensitizers. Martini & Bonsignore [1934] found that *l*-ascorbic acid reduced methylene blue to its leuco base when exposed to the light of an ordinary electric lamp. Kon & Watson [1936] observed that on exposure to visible light of short wave length (blue and violet) *l*-ascorbic acid in milk was oxidized to dehydroascorbic acid. A probable explanation for this phenomenon is found in the observation made by Hopkins [1937; 1938] that lactoflavin and to a lesser extent lumichrome could act as sensitizers in the oxidation of *l*-ascorbic acid when exposed to solar radiation.

In this communication experiments are described which show that light is capable of producing a chemical change in *l*-ascorbic acid even in the absence of sensitizers or catalysts. This photochemical action is, however, restricted to the ultraviolet region of the spectrum. It further appears that the photochemical change takes place not only with but also apparently without the intervention of oxygen. So far as cause and mechanism are concerned this reaction, therefore, differs from those observed by the above-mentioned workers.

Technique

All the solutions were made up in phosphate buffer (*p*H 7) prepared with quartz-distilled water. This *p*H was employed in preference to *p*H 7·4, since it was more favourable to the stability of dehydroascorbic acid. The solutions (50 ml.) were exposed in glass or quartz round-bottomed flasks, according to the nature of the experiment, to a Hewittic Y 2 mercury vapour lamp at a distance of one foot. Control experiments carried out in the dark were kept at 37° since this was the temperature reached by the solutions, owing to heat emitted by the lamp, after one hour's exposure. In the anaerobic experiments the flasks were previously exhausted and filled with nitrogen three times and finally exposed in an atmosphere of this gas. Dehydroascorbic acid was determined by reducing the solutions with hydrogen sulphide at *p*H 3 after the addition of glacial acetic acid, one drop of 1 % NaCN and one drop of octyl alcohol. The hydrogen sulphide was removed in the usual way by a current of nitrogen. When sensitizers were present in solution the displacement of the hydrogen sulphide was carried out in the dark.

The behaviour of l*-ascorbic acid on exposure to visible and ultraviolet light*

The decomposition of *l*-ascorbic acid by the direct action of ultraviolet light can be conveniently demonstrated by following up the disappearance of *l*-ascorbic acid in solutions exposed to a mercury vapour lamp in quartz and in glass vessels

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with and without sensitizers. In the experiment to be described *l*-ascorbic acid solutions in phosphate buffer at *p*H 7 were therefore irradiated in quartz and glass flasks in the absence of a sensitizer and in glass flasks in the presence of methylene blue and of lactoflavin. In addition, a solution of *l*-ascorbic acid in a glass flask was kept in the dark at 37° to serve as a control. At suitable intervals samples were removed and titrated at *p*H 3 with indophenol, whilst a duplicate of each sample was treated with hydrogen sulphide as described above. It was found possible to determine *l*-ascorbic acid in the presence of methylene blue, since the latter does not interfere with the end point; a very slight excess of indophenol produces a purple coloration in the blue solution. The results in Table I show that in the absence of a sensitizer the *l*-ascorbic acid in the glass

Table I. *Effect of exposure of* l*-ascorbic acid to mercury vapour lamp*

mg. of *l*-ascorbic acid per 100 ml.

	Exposure of *l*-ascorbic acid to mercury vapour lamp									
	In the dark		In quartz		In glass		In glass in the presence of methylene blue (0·5 mg./100 ml.)		In glass in the presence of lactoflavin (0·5 mg./100 ml.)	
Time min.	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	3·95	4·00	3·88
15	4·00	4·00	2·50	3·00	4·00	4·00	2·00	3·57	0·80	2·70
30	3·98	4·00	0·68	1·97	4·00	4·00	0·60	2·70	0·00	1·92
60	3·97	4·00	0·00	0·78	3·80	3·75	0·00	1·20	0·00	0·90

flask, i.e. the solution which was exposed to visible light only, was not decomposed to any appreciable extent after 60 min. exposure, thus resembling the *l*-ascorbic acid solution kept in the dark. In contradistinction to this the solution in the quartz flask which was exposed both to visible and ultraviolet light simultaneously showed a progressive diminution in the *l*-ascorbic acid content. As was to be expected in the presence of methylene blue or of lactoflavin the *l*-ascorbic acid gradually disappeared when exposed even to visible light. It will also be noted that there was a formation of dehydroascorbic acid simultaneously with the disappearance of the *l*-ascorbic acid in each case.

The decomposition of l*-ascorbic acid by ultraviolet light under anaerobic conditions*

In the preceding experiment the exposure to the ultraviolet light took place in the presence of air and, as was seen, there was a simultaneous formation of dehydroascorbic acid in spite of the absence of a sensitizer. An experiment will

Table II. *Effect of exposure of* l*-ascorbic acid to mercury vapour lamp under anaerobic conditions*

mg. of *l*-ascorbic acid per 100 ml.

	In the dark		Exposed to mercury vapour lamp	
Time min.	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
0	4·00	4·00	4·00	4·00
15	4·00	4·00	2·20	2·49
30	3·90	4·00	0·90	1·13
60	3·80	3·85	0·00	0·27

now be described in which the irradiation of an *l*-ascorbic acid solution took place in the absence of atmospheric oxygen. The method employed to ensure this condition has already been described. The results obtained (Table II) leave no doubt that even in the presence of such traces of oxygen as may have been left there was a photochemical decomposition of *l*-ascorbic acid. Furthermore no formation of dehydroascorbic acid could be demonstrated in this case. The slight increase in the titration after treatment with hydrogen sulphide was of a similar order to that observed in the control solution kept in the dark.

The stability of dehydroascorbic acid to ultraviolet light under anaerobic conditions

Dehydroascorbic acid was prepared immediately before use by oxidizing *l*-ascorbic acid with the theoretical amount of potassium iodate in the presence of potassium iodide and hydrochloric acid. 1 ml. of a 0·2 % solution was then added to 49 ml. of phosphate buffer (*p*H 7·0). The solution was exposed to the mercury vapour lamp in a quartz flask under anaerobic conditions. The determinations were carried out by titrating with indophenol the ascorbic acid formed after reduction with hydrogen sulphide. Dehydroascorbic acid is not very stable even under anaerobic conditions in the dark, most probably because of the gradual opening of the ring in the molecule [cf. Herbert *et al.* 1933], but, as will be seen from Table III, the deterioration in the exposed solution did not take place at a

Table III. *Effect of exposure of dehydroascorbic acid to mercury vapour lamp under anaerobic conditions*

mg. of *l*-ascorbic acid per 100 ml. after treatment with H_2S

Time (min.)	In the dark	Exposed to mercury vapour lamp
0	3·66	3·66
15	2·77	2·52
30	1·92	1·78
60	1·25	0·96

much higher rate than in the control solution kept in the dark. It may therefore be concluded that ultraviolet light has no very marked deleterious effect on dehydroascorbic acid in the absence of oxygen. This result is in consonance with the fact that a freshly made up solution of this compound shows no selective absorption in the ultraviolet region [Herbert *et al.* 1933].

The inability of lactoflavin to sensitize decomposition of l*-ascorbic by ultraviolet light under anaerobic conditions*

Although lactoflavin catalyses the oxidation of *l*-ascorbic acid in the light it was nevertheless desirable to ascertain whether it influenced the anaerobic decomposition of the compound by ultraviolet light. The results are given in Tables IV and V. They suggest that in the absence of oxygen lactoflavin has very little, if any, influence on the rate of the decomposition of *l*-ascorbic acid. It is true that a somewhat greater disappearance of *l*-ascorbic acid was observed when the solutions were exposed to the mercury vapour lamp in the presence than in the absence of the sensitizer. This, however, was undoubtedly due to the fact that in spite of all the precautions taken, a certain very short exposure to light and oxygen could not be avoided during the removal and titration of the samples. In the presence of oxygen lactoflavin, as was to be expected, accelerated markedly the oxidation of the *l*-ascorbic acid.

Table IV. l-*Ascorbic acid exposed to mercury vapour lamp under anaerobic conditions in the presence and absence of lactoflavin*

mg. of *l*-ascorbic acid per 100 ml.

	In the dark				Exposed to mercury vapour lamp			
	Without lactoflavin		With lactoflavin (0·5 mg./100 ml.)		Without lactoflavin		With lactoflavin (0·5 mg./100 ml.)	
Time min.	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	3·89
15	4·00	4·00	4·00	4·00	2·25	2·56	1·90	2·40
30	3·90	4·00	3·90	3·95	1·14	1·43	0·96	1·30
60	3·80	3·80	3·75	3·90	0·10	0·35	0·00	0·25

Table V. l-*Ascorbic acid exposed to mercury vapour lamp under aerobic conditions in the presence and in the absence of lactoflavin*

mg. of *l*-ascorbic acid per 100 ml.

	In the dark				Exposed to mercury vapour lamp			
	Without lactoflavin		With lactoflavin (0·5 mg./100 ml.)		Without lactoflavin		With lactoflavin (0·5 mg./100 ml.)	
Time min.	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	4·00
15	4·00	4·00	4·00	4·00	1·96	3·10	0·00	2·70
30	3·90	4·00	3·90	4·00	0·20	1·08	0·00	1·67
60	3·80	4·00	3·70	3·75	0·00	0·14	0·00	0·30

The influence of acidity on the decomposition of l-*ascorbic acid by ultraviolet light*

It is now well known that the atmospheric oxidation of *l*-ascorbic acid in the presence of metallic catalysts proceeds more slowly in acid solution than in neutral or alkaline solution and therefore a retardation in the disappearance of *l*-ascorbic acid when exposed to ultraviolet light in the presence of air in acid solution was to have been anticipated. It was, nevertheless, of interest to study the effect of *p*H on the anaerobic photochemical decomposition. *l*-Ascorbic acid solutions buffered at *p*H 3 and *p*H 7 were therefore exposed to ultraviolet light in the presence and in the absence of air. The results (Table VI) show that not

Table VI. *The influence of acidity upon the decomposition of* l-*ascorbic acid exposed to mercury vapour lamp*

mg. of *l*-ascorbic acid per 100 ml.

	Exposed to mercury vapour lamp							
	Anaerobic conditions				Aerobic conditions			
	*p*H 3·0		*p*H 7·0		*p*H 3·0		*p*H 7·0	
Time min.	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
0	3·80	3·80	3·80	3·82	3·80	3·80	3·80	3·82
15	2·68	3·44	2·22	2·52	2·44	3·47	2·16	3·09
30	2·04	3·32	0·90	1·28	0·86	2·94	0·60	1·97
60	1·20	2·84	0·00	0·40	0·30	2·07	0·00	1·32

only under aerobic but also under anaerobic conditions the lower *p*H had a retarding influence on the disappearance of the *l*-ascorbic acid. Corresponding control solutions kept in the dark showed hardly any decomposition. It is of interest to note that after treatment with H_2S there was an appreciable rise in the indophenol-reducing capacity at *p*H 3 even under anaerobic conditions.

Summary and conclusions

The results reported in this communication show that *l*-ascorbic acid, but not dehydroascorbic acid, undergoes photochemical decomposition when exposed to the light of the ultraviolet region of the spectrum without the intervention of oxygen. It may be argued that the disappearance of the *l*-ascorbic acid in the anaerobic experiments was due to the presence of the last traces of oxygen which were not removed after repeated exhaustion. The formation of dehydroascorbic acid owing to its comparative stability at *p*H 7 (Table III) should then have been detected, which as will be seen from Table II was not the case. Furthermore, we have observed that on exposing higher concentrations of *l*-ascorbic acid under anaerobic conditions the quantities which were decomposed were far in excess of those which could have been oxidized by any traces of oxygen which were left in solution. The following example will illustrate the point. A buffered solution (*p*H 7) of 50 ml. containing 50 mg. of *l*-ascorbic acid was exposed to the mercury vapour lamp anaerobically for 6 hr. under the above conditions. 22 mg. of the acid disappeared during this period. If the disappearance of the compound were due entirely to oxidation, 1·40 ml. of oxygen would have been necessary—a quantity which could not possibly have been present in solution even without previous exhaustion. In the presence of air, however, the oxidation of *l*-ascorbic acid is undoubtedly catalyzed by this light. Under both conditions the reactions proceed without the aid of a sensitizer. Lactoflavin which acts as a photocatalyst in the oxidation of *l*-ascorbic acid by visible light has no action on the photochemical change of this compound in the absence of oxygen. Acidity which retards the oxidation of *l*-ascorbic acid also retards, although to a lesser extent, its photochemical decomposition when oxygen is excluded. These observations taken in conjunction with the fact that the oxidation of *l*-ascorbic acid is catalyzed by visible light bring into prominence the complexity of the nature of the decomposition of *l*-ascorbic acid by light.

It may be observed in this connexion that the slightly increased decomposition of ascorbic acid recorded by Kon & Watson [1936] when milk was exposed in quartz rather than in glass vessels, was presumably due to the *direct* action of ultraviolet light. The greater part of the decomposition of the acid in the quartz vessel was, however, brought about probably in the same way as in the glass vessel, namely, as suggested by Hopkins [1938], by the action of visible light sensitized by the lactoflavin of the milk.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant. We should also like to express our thanks to Messrs Roche Products Ltd. for a gift of *l*-ascorbic acid and lactoflavin.

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CCIV. STUDIES ON CAROTENOIDS

V. GAZANIAXANTHIN

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THREE xanthophylls of the formula $C_{40}H_{56}O$ are known, of which cryptoxanthin [Kuhn & Grundmann, 1933] is related to β-carotene, rubixanthin [Kuhn & Grundmann, 1934] to γ-carotene, and lycoxanthin [Zechmeister & Cholnoky, 1936] to lycopene. In this paper we describe a fourth xanthophyll with one oxygen atom, which has been isolated from the petals of *Gazania rigens*, and which, judging from its behaviour, is related to γ-carotene and rubixanthin.

The flowers of *G. rigens* are very interesting from the biochemical standpoint since the great majority of its carotenoids belong to the γ-carotene series, which rarely occurs in other plants. The greater part of the carotenoids of this plant appears in the "carbohydrate fraction", which includes also the xanthophylls with one oxygen atom. This fraction contains four different carotenoids, three of which are present in about equal amounts, and the fourth—probably γ-carotene—only in much smaller quantities. All these four substances have practically the same absorption spectrum as γ-carotene; they can be separated chromatographically.

Chromatographic analysis of the saponified lipoid extracts of *Gazania* flowers gives an upper zone of rubixanthin, which can easily be obtained in a pure crystalline state. The second coloured zone, separated from the first by a large colourless one, contains the new xanthophyll gazaniaxanthin. It is accompanied by another unknown carotenoid with the same absorption spectrum, adsorbed closely under the zone of gazaniaxanthin, and difficult to separate from it chromatographically. This substance has not yet been obtained crystalline. Below this zone, follows a small zone of γ-carotene.

Gazaniaxanthin crystallizes from a mixture of benzene-methyl alcohol (1 : 4) in brilliant rectangular leaflets of a deep red colour, whose macroscopic and microscopic appearance is very similar to that of cryptoxanthin. The crystals contain methyl alcohol from which they can be freed only with difficulty. In this respect gazaniaxanthin is rather different from rubixanthin, which crystallizes without methyl alcohol, in red-brown needles of a much lighter colour.

Gazaniaxanthin is readily soluble in benzene and light petroleum, very sparingly soluble in methyl alcohol. During partition between light petroleum and methyl alcohol, gazaniaxanthin behaves like rubixanthin. It melts at 136–137° (uncorr., in evacuated tube).

The spectral behaviour of gazaniaxanthin is very similar to that of γ-carotene and rubixanthin, the absorption maxima being slightly displaced to shorter wave-lengths.

Absorption maxima of gazaniaxanthin[1]

	mμ
In carbon disulphide	530, 495·5, 463
In light petroleum (B.P. 80°)	494, 461, 433·5
In hexane	492·5, 460, 432
In benzene	508, 476·5
In absolute ethyl alcohol	494·5, 462·5

Elementary analyses of gazaniaxanthin agree with the composition $C_{40}H_{54}O$ or $C_{40}H_{56}O$. The presence of an alcoholic hydroxyl group was demonstrated by the formation of an acetyl derivative with acetic anhydride in pyridine solution.

Gazaniaxanthin in the crystalline state binds solvents and ash tenaciously and in spite of repeated crystallizations we did not succeed in obtaining absolutely ash-free preparations. We believe that this fact is the cause of the ease with which solutions and crystals of this carotenoid undergo autoxidation, whereby it is transformed in the course of a few days even in the cold into a colourless crystalline substance.

In its chromatographic behaviour gazaniaxanthin is intermediate between rubixanthin and lycopene. A mixture of these three substances can be easily separated in the alumina column.

The elucidation of the chemical structure of gazaniaxanthin will be possible only after further investigation. Kuhn & Grundmann [1934] have demonstrated that rubixanthin is a derivative of γ-carotene containing a hydroxyl group in the β-ionone ring; it is suggested that gazaniaxanthin may be an isomeride of this having the hydroxyl group in the aliphatic side chain, like lycoxanthin and lycophyll [Zechmeister & Cholnoky, 1936]. The correctness of this supposition can be tested by a biological assay, as a substance of the constitution proposed should be active as provitamin A in contrast to rubixanthin.

Experimental

We are obliged to Dr Mendonça of the Botanical Institute of this University for the following notes about *Gazania* plants.

Gazania rigens, of the family of Compositae, is native to Africa. It is cultivated in Portugal in the open air, whereas in the north of Europe it can only live in hot houses. The plant, though abundant in flowers, does not fructify in Portugal, and the flowers used for our investigation are from a specimen brought to the Botanical Garden of Coimbra about a century ago, and which has since then reproduced only asexually by means of suckers.

The plant flowers from March to the end of May, only a small part flowering at a time. We collected the flowers once a week. The plant contains a latex-like sap, which is exceptionally rich in lipoids.

The petals (about 200 g. collected in a week), are separated and dried in a stream of warm air at 50° for several hours. The dry material (40 g.) is finely pulverized in a mortar, and then exhaustively extracted with hot light petroleum (B.P. 30–50°) in an atmosphere of CO_2.

The solution, on standing in the ice box, deposits crystals of free xanthophyll and a part of the resinous substance. After filtering, the solution is evaporated *in vacuo* at a low temperature until it begins to foam. The residue is dissolved in 150 ml. of hot absolute ethyl alcohol. This solution, during slow refrigeration, precipitates a considerable quantity of colourless resinous substance, which is

[1] Determined with a Hilger prism-spectroscope and a copper sulphate-ammonia filter.

filtered off after about half an hour. It is important not to cool this alcoholic solution too much, or to let it stand for too long, as otherwise a part of the carotenoid pigments also precipitates.

The solution is filtered and 50 ml. of pure thiophen-free benzene and 5 ml. of saturated aqueous KOH are added and the homogeneous clear mixture is kept at room temperature (22°) in well filled stoppered bottles for 2 days. Then 300 ml. of light petroleum and 50 ml. of water are added and the mixture is agitated. Nearly all the colouring matter remains in the upper layer. The alkaline alcoholic layer is discarded as it contains much resinous matter and only a little xanthophyll.

The light petroleum solution is thrice extracted, each time with 50 ml. of methyl alcohol (90 %), which dissolves a quantity of xanthophyll. The solution is then washed several times with water, to remove the methyl alcohol, dried with sodium sulphate and filtered. When kept in the ice box a quantity of colourless substances (various sterols) separate, which have been obtained in a pure state.

It proved of special importance for the chromatographic separation of the carotenoids present in the light petroleum solution to choose an adsorbing substance which is not too active, as otherwise in consequence of the many colourless substances present, and the relatively small differences in adsorbing affinity of the pigment, no sufficient separation can be obtained. We have found that, though separation may be effected with calcium hydroxide, this substance is not very appropriate because of its bad filtering qualities and the indistinct formation of the zones. The best material for the separation of the carotenoids of *Gazania* flowers is alumina. We used Merck's ordinary aluminium oxyd. puriss. anhydr., which, as it was still too active, was moistened with light petroleum and kept in the air for 1 day.

The solution is filtered through a column 15 cm. high and 5 cm. in diameter, without suction, and the chromatogram is developed with a mixture of benzene-light petroleum (1 : 3). Six zones develop. The first small, deep red, zone still contains a little xanthophyll, which was recognized to be pure lutein. Then follows a deep brown-red zone 5 mm. thick, which contains rubixanthin. The third zone, 3–4 cm. thick contains only colourless substances. Then comes a violet-red zone 4 mm. thick, immediately followed by another brown-red zone of double this thickness. From the former we have isolated gazaniaxanthin, while the latter has not yet furnished a crystalline coloured substance. The isolation and crystallization of gazaniaxanthin depends essentially on the sharp separation of the upper uncoloured and the lower coloured zones.

At some distance from the fifth zone, follows a small yellow ring which passes more rapidly to the filtrate and which shows the bands of γ-carotene. A very small first part of the yellow filtrate shows absorption bands of β-carotene.

Rubixanthin. The zone of the chromatogram containing rubixanthin is eluted with light petroleum containing 1 % of methyl alcohol and evaporated *in vacuo*. The residue is dissolved in 10 ml. of hot light petroleum and kept for 2 days in the ice box. Deep red crystals and a red oil separate. The precipitate is filtered and the crystals remaining on the filter are freed from the oil by washing with cold light petroleum which contains a few ml. of absolute ethyl alcohol. The crude crystals have M.P. 145°. They are dissolved in a mixture of 15 ml. methyl alcohol and 10 ml. ether, and the solution evaporated on the water bath until crystallization begins. The solution is kept at room temperature for half an hour, then in the ice box overnight. The crystals obtained are once

more crystallized from a mixture of benzene-methyl alcohol (1 : 3). 5 mg. of fine red-brown needles are obtained with M.P. 160° (uncorr., in evacuated tube). Absorption bands in CS_2: 533·5, 498, 465 mμ; in light petroleum (B.P. 80°) 496, 461 mμ. From the mother liquors more rubixanthin of M.P. 150–153° was obtained.

Gazaniaxanthin. The zone of the chromatogram which contains the gazaniaxanthin is carefully separated from the other zones above and below and eluted with light petroleum containing 1 % of methyl alcohol. The solution is evaporated to dryness *in vacuo*. The residue solidifies within a few seconds. It is dissolved in the minimum necessary amount of hot light petroleum (5 ml. approximately) filtered and kept in the ice box. After half an hour gazaniaxanthin begins to crystallize in brilliant orange leaflets. After 20 hr. the crystals are filtered, and twice crystallized from a mixture of benzene-methyl alcohol (1 : 4). From this mixture the carotenoid crystallizes in brilliant rectangular crystals of a deep red colour with strong dichroism, and birefringence. From a mixture of light petroleum and methyl alcohol brown-red crystals of more irregular shape are obtained. Both these crystals contain methyl alcohol, of which the greater part is lost during heating *in vacuo* to 80–90°. The M.P. determined in a circular bath of sulphuric acid was found at 136–137° (uncorr., in evacuated tube).

During heating in high vacuum at 80° the carotenoid lost 0·92, 0·89 mol. of methyl alcohol.

Found (for dry and ash-free substance): C, 87·12, 87·39 %. H, 10·75, 10·73 %. Calculated for $C_{40}H_{56}O$: C, 86·89 %; H, 10·22 %. $C_{40}H_{54}O$: C, 87·27 %; H, 9·90 %.

Gazaniaxanthin acetate. 10 mg. of gazaniaxanthin were dissolved in 23 ml. of pyridine and 2 ml. of acetic anhydride added. The mixture was kept for 1 day in a well-filled, stoppered bottle. Then 75 ml. of benzene were added and the solution shaken many times with water to remove all pyridine. The solution was dried with sodium sulphate, filtered and concentrated *in vacuo* to 3 ml. 9 ml. of absolute methyl alcohol were added and the mixture kept in the ice box. In the course of several days, the acetate crystallized. It was twice recrystallized from a mixture of benzene-methyl alcohol. The acetate tends to crystallize slowly, in the course of several days only.

Gazaniaxanthin acetate is more soluble in benzene and light petroleum than the free xanthophyll. It crystallizes from a mixture of benzene-methyl alcohol in thick star-shaped orange needles. From light petroleum-methyl alcohol fine, long, needles appear, frequently curved; M.P. 83–85° (uncorr., in evacuated tube) with strong foaming, probably losing solvent.

The absorption spectrum is identical with that of gazaniaxanthin; in CS_2: 530, 494 mμ; in benzene 508, 476, 445 mμ.

Xanthophylls. The methyl alcohol extracts obtained after saponification are, after addition of 10 ml. of light petroleum, carefully diluted with water. Crystalline xanthophyll precipitates. The ethereal solution of this crude xanthophyll gives a faint blue colour with conc. HCl, which, however, disappears after further crystallizations. The xanthophyll is recrystallized from a mixture of chloroform-light petroleum, then twice from a mixture of methyl alcohol-ether. A small portion of "leaf xanthophyll" is obtained; M.P. 204° (uncorr.). Absorption bands in CS_2: 509, 478 mμ. During chromatographic adsorption of the xanthophyll on to calcium carbonate, two zones are observed, the upper orange yellow, the lower light yellow. We have not tried to isolate the components in a pure state.

From the first zone of the chromatogram of the hydrocarbon fraction we obtained by elution with methyl alcohol a solution, from which after evaporation

in vacuo and two crystallizations from a mixture of methyl alcohol-ether, pure lutein was isolated. It crystallized in yellow needles, which, in contact with the methyl alcohol, were transformed in the course of several days into deep red crystals, which contained methyl alcohol. M.P. 195° (uncorr., in evacuated tube). Absorption bands in CS_2 508, 476, 445 mμ.

Summary

1. A new xanthophyll with one oxygen atom, gazaniaxanthin, has been isolated from the flowers of *Gazania rigens*. Its physical and chemical properties are similar to those of rubixanthin.

2. In the flowers of *Gazania rigens* there occur, besides gazaniaxanthin, rubixanthin, lutein and "leaf xanthophyll", which were isolated in a pure crystalline state, and probably γ-carotene. Another carotenoid with the same absorption spectrum as γ-carotene could not be isolated in the crystalline state.

The author wishes to express his thanks to Prof. Dr A. de Morais-Sarmento, Director of this Laboratory, for his great interest during the course of this work. He is also indebted to Prof. Dr R. G. Couceiro da Costa, Director of the Chemical Laboratory for placing at his disposal the facilities of the Institute.

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CCV. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST

V. THE SYNTHESIS OF FAT FROM ACETIC ACID: THE INFLUENCE OF METALLIC IONS ON CARBOHYDRATE AND FAT STORAGE

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(*Received 8 July 1938*)

WHEN yeast is suspended in oxygenated water, part of its reserve carbohydrate is transformed into lipoid; if, however, it be incubated in an oxygenated solution of sodium acetate, the amount of lipoid formed is greater and is indubitably formed at the expense of the acetate molecule [Smedley-Maclean & Hoffert, 1926; Wieland & Wille, 1935].

There appeared to be three possible ways in which this synthesis could be effected: (1) the fatty acid chains might be built up at the expense of the acetate molecules; (2) the reserve carbohydrate present in the cell might furnish some degradation product capable of reacting with the acetate molecules; (3) carbohydrate might be formed from the acetate and converted into fat.

It was therefore interesting to know whether any simple carbon compound formed by the condensation of acetic acid could be utilized for fat formation. Butyric, acetoacetic and β-hydroxybutyric acids had already been tested with negative results [Smedley-Maclean & Hoffert, 1926]; succinic acid, formed in a yield of 10% when yeast is incubated in an oxygenated acetate solution and citric acid, formed from acetate in presence of Ba^{++} ions [Wieland & Sonderhoff, 1932], have now been tested and found to be ineffective as are also fumaric and malic acids. Acetoin, synthesized by yeast in large yields when acetaldehyde is added to a fermenting sugar solution [Neuberg & Reinfurth, 1923], was also inactive. The results of our experiments are set forth in Table I.

Yeast incubated in the solutions tested showed about the same fat content as yeast which has been incubated in oxygenated water, with three exceptions, diacetyl, methylvinyl ketone and sodium sorbate, in all of which a system of conjugated double bonds is present; this structure apparently inhibits the transformation of reserve carbohydrate into fat normally taking place when yeast is incubated in oxygenated water.

So far, therefore, it has not been possible to find evidence that any simple carbon compound containing 4–6 carbon atoms, which might be regarded as a condensation product of acetic acid, can act as an intermediate substance in the synthesis of fat.

In support of the hypothesis that fatty acids might be formed by the interaction of the acetate molecule with some degradation product of carbohydrate, experiments carried out by Stephenson & Whetham [1922] were recalled in which the addition of sodium acetate to the glucose solution in which the Timothy grass bacillus was incubated resulted in an increase of the amount of fat stored.

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Table I

Showing mg. of fat and carbohydrate contained in 10 g. yeast after incubation in an oxygenated solution of substance to be tested. The figures after incubation of 10 g. of the same sample of yeast in oxygenated Na acetate solution are given for comparison. The phosphate present in the solution was 1·27% as a mixture of sodium hydrogen phosphates *p*H 6·8. CH = carbohydrate; Lipoid = ether-soluble material. The methods of estimation were those used by Smedley-Maclean & Hoffert.

				Yeast after incubation in					
		Original yeast		(1) Solution Na acetate *N*/14		(2) Solution substance		(3) Solution substance with $PO_4^{\equiv}$	
Substance	%	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid
Na salt of:									
Citric acid	0·52	—	84	237	190	250	117	—	—
Succinic acid	0·44	—	86	250	214	377	134	310	153
	0·22	760	82	165	123	310	137	250	144
Fumaric acid	0·43	595	83	232	195	—	—	165	153
Maleic acid	0·43	—	—	—	—	—	—	200	145
Crotonic acid	0·32	—	—	302	203	245	109	—	—
Laevulic acid	1·00	560	86	185	207	—	—	195	128
Acetoin	0·32	637	84	237	190	—	—	167	134
	0·50	842	89	167	242	—	—	170	213
	0·50	750	104	180	234	—	—	192	173
	0·50	742	84	230	220	—	—	222	175
	0·50	645	80	192	198	—	—	192	141
	0·64	850	89	325	230	280	143	192	157
	0·78	742	82	345	185	—	—	197	151
2:3-Butylene glycol	0·66	820	87	192	230	415	128	295	153
Methylethyl ketone	0·30	—	80	—	173	—	—	—	137
Substances containing conjugated double bonds									
Diacetyl	0·65	750	91	185	196	645	84	385	81
Methylvinyl ketone	0·40	750	104	180	234	—	—	267	96
	0·40	742	84	230	220	—	—	360	77
Sodium sorbate	1·00	712	87	187	243	292	74	150	87
	1·00	802	87	212	201	337	74	187	98

The effect of adding sodium acetate to a pure glucose solution in which yeast was incubated was to produce no increase in lipoid but to bring about a considerable lowering of the carbohydrate content.

In Stephenson & Whetham's experiments the medium in which the Timothy grass bacillus was grown contained glucose, $CaCO_3$ to neutralize any free acids produced, and inorganic salts, ammonium salts furnishing a supply of nitrogen. As we were concerned only with the transformation of glucose into lipoid in the existing cells and desired to exclude the formation of new cells a nitrogen supply had to be eliminated but we proceeded to examine the effect of the addition of $CaCO_3$ to the glucose medium in which the yeast was incubated.

When $CaCO_3$ was added to the glucose solution in which the yeast was incubated the amount of lipoid fell to about two-thirds of that formed in the absence of $CaCO_3$. Addition of sodium acetate to the glucose and $CaCO_3$ medium produced, however, a slight rise in the amount of lipoid stored (cp. Table II); the carbohydrate content showed no significant alteration.

The addition of $CaCO_3$ to the glucose solution produced a distinct sedimentation of the yeast, and it seemed possible that this mechanical effect might have interfered with the formation of lipoid for which free oxygenation is essential. The replacement of the $CaCO_3$ by kieselguhr both in its original state and after washing with HCl produced fairly rapid settling of the yeast and in its

Table II

Showing the effect of $CaCO_3$ on fat and carbohydrate storage by yeast incubated in oxygenated solutions of glucose and of glucose and sodium acetate respectively. CH = carbohydrate. Figures represent mg./10 g. yeast

Original		Glucose		Glucose + $CaCO_3$		Glucose + Na acetate		Glucose + $CaCO_3$ + Na acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
78	655	—	—	216	667	—	—	253	640
115	460	452	540	315	610	—	—	359	720
80	—	406	—	—	—	381	—	—	—
98	700	415	550	—	—	415	400	—	—
79	550	415	600	—	—	408	350	—	—
89	720	403	660	—	—	335	470	—	—
74	400	326	560	223	520	—	—	231	450
Mean: 88	550	402	580	251	600	385	370	281	600

original state a fall of stored lipoid similar to that produced by $CaCO_3$. After the kieselguhr had been well washed with acid, no inhibitory effect was produced on the lipoid formation: the washings contained Ca^{++} and Mg^{++} removed from the original kieselguhr. These results suggested that Ca^{++} or Mg^{++} and not the sedimentation was the inhibiting factor.

Table III

Showing mg. lipoid and carbohydrate stored in 10 g. yeast after incubation in oxygenated 2% glucose solution to which kieselguhr had been added.

Original		After incubation in					
		(*a*) 2% glucose solution		(*b*) 2% glucose solution with kieselguhr		(*c*) 2% glucose solution with *N*/14 Na acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
		(1) Unwashed					
89	670	309	745	239	862	305	—
96	672	392	580	282	560	396	457
		(2) Washed with HCl					
88	680	371	652	345	565	313	600
80	642	415	457	400	565	391	402

Table IV

Showing mg. lipoid in 10 g. yeast after suspension in oxygenated solutions of 2% glucose to which had been added (*b*) $CaCO_3$, (*c*) sodium acetate + $CaCO_3$, (*d*) sodium glycollate + $CaCO_3$, (*e*) sodium succinate + $CaCO_3$.

Original yeast Lipoid	(*a*) Glucose Lipoid	(*b*) Glucose + $CaCO_3$ Lipoid	(*c*) Glucose, $CaCO_3$ and *N*/14 Na acetate Lipoid	(*d*) Glucose, $CaCO_3$ *N*/14 Na glycollate Lipoid	(*e*) Glucose, $CaCO_3$ and *N*/14 Na succinate Lipoid
102	—	227	—	381	—
79	—	329	—	397	—
78	—	216	253	276	—
84	—	195	—	256	278
115	452	315	359	—	—
74	326	223	231	—	—

The increase of lipoid produced by the addition of sodium acetate to the glucose-$CaCO_3$ medium was not specific for the acetate but was produced by the addition of other organic Na salts to the medium, and probably indicated an antagonistic effect of the Na^+ and Ca^{++} ions.

The inhibitory effects produced on lipoid storage by the addition of NaCl, $CaCl_2$ and $MgCl_2$ respectively to the glucose medium are set forth in Table V.

Table V

Showing the lipoid and carbohydrate (CH) content in mg. per 10 g. yeast after incubation in 2% glucose solution with and without the addition of chlorides. Medium oxygenated for 24 hr.

Original yeast		Medium											
		(*a*) 2% glucose		(*b*) 2% glucose + NaCl			(*c*) 2% glucose + $CaCl_2$			(*d*) 2% glucose + $MgCl_2$			
Lipoid	CH	Lipoid	CH	NaCl	Lipoid	CH	$CaCl_2$	Lipoid	CH	$MgCl_2$	Lipoid	CH
100	590	404	580	*N*/14	400	420	*N*/14	272	500	*N*/14	261	580
100	380	392	470	*N*/14	406	340	*N*/14	242	330	*N*/14	244	370
92	660	443	747	—	—	—	*N*/350	280	727	—	—	—
95	610	401	660	—	—	—	—	—	—	—	—	—
—	740	429	630	—	—	—	*N*/350	275	640	*N*/14	277	410
							N/700	333	700	*N*/140	298	550
							N/140	242	630	*N*/1400	371	580
98	740	432	610	—	—	—	*N*/14	228	450	—	—	—
							N/140	232	600	—	—	—
							N/1400	358	—	—	—	—

The *p*H of the medium at the end of these experiments was in each case approximately between 3 and 5. The variation in *p*H seemed to have little effect on the power of lipoid synthesis.

These experiments brought home to us the fact that the nature of the metallic ions present in the medium exercised a considerable influence on the lipoid storage. Further investigation of the comparative effects of the ions, K^+, Na^+, Ca^{++} and Mg^{++}, confirmed that both Ca^{++} and Mg^{++} exercise a very marked inhibition on the formation of lipoid material from glucose, Na^+ being almost without action; on the other hand, the presence of Na^+, Ca^{++} or Mg^{++} reduced, to some extent, the carbohydrate content.

An instance of the marked inhibitory effect of Ca^{++} and Mg^{++} was provided by some experiments in which yeast was incubated in media consisting respectively of glucose and commercial fructose solutions. The yeast incubated in the fructose solution had a very low lipoid content which showed no diminution when $CaCO_3$ was added to the medium and was much lower than the values previously recorded in similar experiments [Smedley-Maclean & Hoffert, 1923]. The sample of commercial fructose gave a good reaction for Ca and Mg but the purified fructose behaved similarly to the glucose.

The effects of K^+, Na^+, Ca^{++} and Mg^{++}, were then compared by incubating yeast in glucose solutions to which their acetates had been separately added. The results are shown in Table VI.

Here also little effect was produced on the amount of fat stored by the presence of K^+ and Na^+, but Ca^{++} and Mg^{++} produced marked diminutions, Mg^{++} apparently having the greatest influence. The inhibitory effect on lipoid storage appeared to be greater in the presence of the chlorides than of the acetates, a result consistent with the view that the inhibition brought about by Ca^{++} and Mg^{++} might be to some extent compensated by the additional lipoid formed from the acetate ions.

Table VI

Showing mg. lipid and carbohydrate (CH) in 10 g. yeast after incubation for 24 hr. in an oxygenated solution of 2% glucose with $N/14$ acetate (0·72%).

Original yeast		Glucose 2%		Glucose 2% +K acetate		Glucose 2% +Na acetate		Glucose 2% +Ca acetate		Glucose 2% +Mg acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
80	710	398	585	—	—	381	395	311	—	—	—
82	760	370	730	—	—	—	—	317	550	287	780
79	550	415	600	—	—	408	350	342	460	309	380
89	722	403	660	—	—	335	470	309	520	289	570
98	700	415	550	—	—	415	400	402	370	325	370
97	530	—	550	—	—	—	—	297	440	—	—
91	—	436	—	—	—	422	—	388	—	399	—
82	420	364	560	392	670	374	440	—	—	306	537

The carbohydrate content of the yeast was also less in the presence of Na^+, Ca^{++} and Mg^{++}, Na^+ being on the whole the most effective inhibitor of the three. Estimations of the residual sugar showed that in the presence of Ca^{++} the amount of residual sugar was higher, the utilization of the sugar having been less effective; Na^+ and Mg^{++} had no perceptible effect.

When yeast was incubated in oxygenated solutions of the pure acetates, the influence of the ions was closely similar to that produced in the glucose-acetate solutions. The presence of K^+ was most favourable for the storage of both lipoid and carbohydrate; unlike Mg^{++}, Na^+ left the lipoid content almost unaffected but like Mg^{++} it produced a decrease in the amount of carbohydrate; Ca^{++} caused a marked lowering of both lipoid and carbohydrate contents.

Incubation of yeast in oxygenated solutions of acetates (N/*14*)

Table VII

Showing mg. lipoid and carbohydrate stored in 10 g. yeast after 24 hr. incubation in acetate solutions, $N/14$.

Original yeast		After incubation in									
		(1) Water		(2) K acetate		(3) Na acetate		(4) Ca acetate		(5) Mg acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
87	700	—	—	227	343	230	248	—	—	185	262
89	843	—	—	266	263	242	168	—	—	—	—
92	825	—	—	266	232	245	230	—	—	—	—
95	523	—	—	231	260	198	180	—	—	—	—
85	650	—	—	—	—	213	240	170	250	—	—
87	620	139	220	—	—	237	280	150	180	191	280
93	700	134	230	—	—	225	260	206	230	199	280
87	790	125	300	—	—	241	340	185	220	193	300
89	740	128	340	—	—	257	350	166	250	—	—
89	670	111	300	—	—	240	320	181	240	—	—

The effect of increasing the concentration of acetate in the medium on the storage products was determined in solutions of the K salt. No further increase was obtained by raising the percentage of acetate from 0·29 to 1·15%.

The effects of the ions upon yeast with low and high reserves of carbohydrate were then compared. Samples of a yeast with a high carbohydrate content were suspended for 24 hr. in oxygenated water, and in oxygenated solutions of Ca and Na acetates respectively. The yeast from the oxygenated water was then

Table VII A

Showing lipoid and carbohydrate contents in mg./10 g. yeast after incubation in K acetate solution.

	Lipoid	CH
Original yeast	92	530
Yeast after incubation in:		
0·03 % acetic acid as K salt	122	250
0·06 % ,,	158	290
0·29 % ,,	209	340
0·58 % ,,	219	310
	191	320
1·15 % ,,	208	270

transferred to the acetate solutions and the effects on carbohydrate and fat storage compared with those in the yeast which had been at once placed in the acetate solutions.

Table VIII

Showing lipoid content in mg. when 10 g. original yeast from brewery were incubated in oxygenated acetate solutions before and after suspension in oxygenated water. ("Run-down" yeast = yeast after suspension in water.)

	Yeast before incubation in acetate solution		Yeast after incubation in Na acetate solution		Yeast after incubation in Ca acetate solution	
Exp. I	Lipoid	CH	Lipoid	CH	Lipoid	CH
Original	89	735	257	350	166	255
Run down	127	480	261	320	215	280
Difference	+38		+4		+49	
Exp. II						
Original	89	670	240	317	181	245
Run down	111	432	236	305	200	265
Difference	+22		−4		+19	

The yeast made substantially the same amount of fat whether it was at once incubated in the Na acetate solution or whether it was first incubated in oxygenated water, and then transferred to the Na acetate solution. On the other hand, the yeast which had been at once added to the Ca acetate solution had a lower fat content than the yeast transferred to the Ca acetate solution after incubation in oxygenated water. A possible explanation was that the Ca, whilst reducing the amount of lipoid formed from acetate, almost entirely inhibited the formation of fat from the reserve carbohydrate. This was, however, not confirmed since yeast incubated in solutions of $CaCl_2$, with and without $CaCO_3$, formed fat just as well as when incubated in oxygenated water. Possibly some product formed from the Ca acetate exercised an inhibitory effect.

Estimation of the residual acetate in the media. Determination of the acetate in the residual media showed that more acetate had disappeared from the Ca (30 %) than from the Na acetate solution (19·5 %). In both cases about twice as much acetate disappeared when the yeast used had already been incubated in water and contained a low carbohydrate reserve (Na acetate, 47·6 %; Ca acetate, 74·3 %).

The effect of phosphate on lipoid formation from acetate. The mean lipoid content in 8 experiments in which 10 g. yeast were incubated in 0·6 % Na acetate solution was 0·218 g.; in 16 experiments in which yeast was incubated in 0·6 % Na acetate solution containing 1·27 % phosphate (as mixed sodium

hydrogen phosphates pH = 6·8) the mean lipoid content was 0·222 g. The addition of phosphate to the acetate solution therefore produced no significant result.

The composition of the lipoid substance collected from a series of parallel experiments in which samples of the same yeast had been incubated in the various media, is shown in Table IX.

Table IX

Medium	Lipoid g.	Saponification value	Unsaponifiable Wt. g.	Unsaponifiable %	Fatty acids Wt. g.	Fatty acids %
(*a*) Original yeast	1·364	148·8	0·464	34·0	0·860	63·0
2% glucose	2·319	160·4	0·595	25·6	1·588	68·5
0·6% Na acetate	1·757	160·4	0·453	25·8	1·292	73·5
(*b*) Glucose	1·277	180·7	0·236	18·5	0·946	74·1
Glucose with Na acetate	1·206	208·9	0·119	9·9	0·921	76·4
Glucose with Mg acetate	0·963	217·8	0·087	9·6	0·768	79·7
Glucose with Ca acetate	1·092	208·7	0·099	9·1	0·880	80·6
(*c*) Glucose	0·727	168·0	0·198	27·3	0·501	68·9
Glucose with K acetate	0·784	155·8	0·226	28·7	0·530	67·6
Glucose with Na acetate	0·748	161·4	0·198	27·1	0·530	70·8
Glucose with Mg acetate	0·612	172·9	0·166	26·4	0·432	70·6

In Exp. (*b*) the addition of the acetate to the glucose medium produced a fall in the unsaponifiable from 18·9 to between 9 and 10%; this result was not confirmed in other experiments. The addition of Na^+, Mg^{++} and Ca^{++} showed, however, a tendency to raise the proportion of fatty acids to unsaponifiable matter.

Detection of pyruvic acid and of acetaldehyde in the residual media on addition of Na^+, Ca^{++} and Mg^{++} to the glucose solution

When 2:4-dinitrophenylhydrazine reagent was added to the residual glucose and glucose-acetate solutions, a marked difference was observed in the amounts of precipitate which separated immediately at ordinary temperature.

Whereas the residual glucose solution generally remained clear or showed only a slight cloudiness, the glucose solutions to which K, Na, Ca or Mg acetates had been added gave quite copious precipitates, usually most marked in the case of the glucose-Mg acetate medium. Replacement of the acetate solutions by chlorides produced somewhat similar precipitates and the accumulation of carbonyl compounds thus appears to be promoted by the presence of K^+, Na^+, Ca^{++} and Mg^{++}.

Quantitative experiments were carried out in which 40 g. of yeast were incubated in 2 l. of an oxygenated 2% glucose solution to which Na, Ca and Mg acetates were respectively added (N/14). To the resulting filtrates excess of 2:4-dinitrophenylhydrazine reagent was added and the resulting precipitates, formed after 10 min. stirring, filtered, dried at 37° and weighed. These were then extracted with Na_2CO_3 solution and the weights of acid and neutral fractions determined.

	2% glucose	2% glucose +Na acetate	2% glucose +Ca acetate	2% glucose +Mg acetate
Wt. total ppt. g.	0·22	1·93	1·82	1·71
Wt. neutral fraction	0·17	1·45	0·95	0·90
Wt. acid fraction	0·05*	0·43	0·83	0·71

* By difference.

The greater part of the neutral fraction consisted of acetaldehyde dinitro phenylhydrazone; the acid fraction consisted mainly of the pyruvic acid derivative identified by the melting point of its ethyl ester.

A number of comparative experiments were carried out in which portions of 1 g. yeast were incubated in oxygenated glucose solutions to which were added the acetate or chloride of K, Na, Ca or Mg; in some cases Mg or Ca carbonate was added to the respective chloride solution to prevent the development of acidity. The results showed considerable variation but the glucose medium to which K, Mg, Ca or Na acetate or chloride had been added always gave a precipitate with the hydrazine reagent. The glucose-acetate (K, Na and Mg) solutions, when tested with sodium nitroprusside, acetic acid and ammonia, usually developed a blue colour showing the presence of pyruvic acid in the solution. The precipitates and pyruvic acid reaction were only marked in media which had been well oxygenated. The glucose-chloride solutions, like the glucose solution, were strongly acid in contrast to the glucose-acetate solutions, the *p*H of which was usually 5–6.

Discussion of results

Influence of metallic ions on fat and carbohydrate metabolism

Evidence already exists that the nature of the ions in the medium exercises an important influence on the fermentation process. Harden [1917] showed that washed zymin was readily activated by acetaldehyde in the presence of K but not of Na phosphate. When glucose is fermented with yeast juice, the rate of fermentation is delayed if the concentration of sodium phosphate exceeds a certain minimum. Meyerhof [1918] pointed out that NaCl and other salts depress both the rate of attainment of the maximum rate of fermentation and the maximum rate attained; Harden & Henley [1921] found that the chlorides and sulphates of Na and K diminished the maximum rate of fermentation of glucose and fructose by zymin and also the rate of attainment of this maximum, an effect not appreciably modified by the addition of acetaldehyde. The rate of decomposition of pyruvic acid was, however, uninfluenced. An inhibitory action of Na^+ on the phosphorylating coenzymes is described in recent work by Ohlmeyer & Ochoa [1937]; the activities of cozymase and adenylic acid as phosphorylating agents in a medium containing Na_2HPO_4 were 1 : 60, but after the addition of a trace of Mn^{++} to the medium the activities of both increased to 200 : 300. Cozymase was much more sensitive to the inhibitory action of Na^+ than was adenylic acid; the ratio of the concentrations of Mn^{++}, Mg^{++}, K^+ and NH_4^+, which removed the inhibitory influence of the Na^+, was 1 : 50 : 1000 : 2000. Our knowledge of the inhibitory action of Ca^{++} seems to rest chiefly on the work of Fernbach & Schoen [1913; 1914; 1920] who found that considerable quantities of pyruvic acid could be isolated from glucose fermented by various yeasts, notably *Mycolevure Duclaux* in the presence of $CaCO_3$. They ascribed the effect not only to the reaction of the medium, since when beer wort was used as the medium no pyruvic acid could be isolated even after the addition of chalk.

In Neuberg's laboratory, pyruvic acid was isolated from the action of yeast juice on Mg hexosediphosphate solution, glycerol being also formed [Kobel & Scheuer, 1930]. The addition of $MgHPO_4$, MgO or Na_2HPO_4 to a 10 % glucose solution in which fresh yeast was fermenting produced similar results. Neuberg & Kobel [1930] concluded that the phosphate ions were not concerned since MgO produced the same effect, and that Mg ions were not essential since Na_2HPO_4 produced a similar result.

The influence of metallic ions on the storage of fat and carbohydrate by yeast in oxygenated glucose media

In our experiments the addition of $CaCl_2$ or $MgCl_2$ to the oxygenated glucose solution, in which yeast was suspended, produced: (*a*) some diminution of carbohydrate storage, rather varying in amount, the effect increasing with the concentration of the salt (Table V); (*b*) marked diminution in the amount of fat stored; (*c*) accumulation of carbonyl compounds in the media to a varying extent.

Similar results were obtained when the acetates of Ca or Mg were added to the glucose solution, less diminution in lipoid storage being produced than by $CaCl_2$ and $MgCl_2$; possibly the acetate had contributed to the fat formation.

The addition of NaCl or Na acetate to the glucose solution markedly inhibited the carbohydrate storage, but unlike Ca^{++} or Mg^{++}, Na^{+} only produced a very slight inhibition of lipoid storage. Carbonyl compounds were also detected in the medium.

The reaction of the medium did not appear to exercise a very important influence on the storage phenomena. The fat and carbohydrate contents were similar in the alkaline Na acetate-glucose and the acid NaCl-glucose media.

Ca^{++} and Mg^{++} exert a strong inhibitory influence on both fat and carbohydrate synthesis from glucose whereas Na^{+} inhibits mainly the carbohydrate synthesis.

The introduction of these three ions especially of Na^{+} or Mg^{++} also produced an accumulation of carbonyl compounds in the oxygenated medium. It seems possible, therefore, that this accumulation may have been connected with the diminution of power to store carbohydrate whether by inhibiting its synthesis or promoting its breakdown.

The presence of metallic ions in the solution appears to exercise an important influence on the reactions of the cell which has hitherto not been sufficiently recognized.

The formation of fat from acetates (*cf. Table VII*)

The influence of K^{+}, Na^{+}, Mg^{++} and Ca^{++} on the formation of fat from acetate in the absence of sugar is very similar to their effect on the formation of fat from sugar. The average increases in the fat content of a sample of yeast after incubation respectively in oxygenated solutions of $N/14$ acetates and water were:

	Acetate of			
Water	K	Na	Mg	Ca
41%	180%	160%	118%	100%

Since the 41% increase after incubation in water represents the fat formed from reserve carbohydrate, it is clear that fat was formed from acetates and that the inhibitory influence of Ca^{++} and Mg^{++} on the process was considerable. The carbohydrate content of the acetate yeasts was only slightly greater than that of the yeast incubated in water, and since the amount of acetate oxidized was greater in the "run-down" yeast it is probable that the acetate exercised a carbohydrate-sparing action. The level of carbohydrate in the yeast from the acetate solutions and from water was much lower than in the original yeast. We can find no convincing evidence that hydrolysable carbohydrate is formed from acetates. Wieland & Wille [1935] found in a yeast incubated in sodium acetate solution an increase of 6% of lipoid and of 0·03% hydrolysable carbohydrate

reckoned on the dry weight of yeast, the latter variation being probably within the limits of accuracy of the experiment. This result is in striking contrast to those obtained by incubating yeast in solutions of alcohol when large increases in the storage of hydrolysable carbohydrate took place. It is therefore possible that when fat is formed from alcohol it passes through the stage of storage carbohydrate and that no direct condensation of alcohol to fatty acid takes place.

The conclusion that fatty acid is synthesized from acetate without going through the intermediate stage of hydrolysable carbohydrate is supported by the work of Sonderhoff & Thomas [1937] on the composition of yeast grown in a solution of trideuteracetate. These workers found that under the conditions of their experiment, no exchange of light and heavy hydrogen took place between the trideuteracetate and the water in which it was dissolved. The absorptions of oxygen by yeast were practically equal in acetate or trideuteracetate solutions when tested in the Warburg-Barcroft apparatus. After shaking in the oxygenated trideuteracetate solution, the deuterium contents of the dried yeast and of its lipoid and carbohydrate were respectively determined. The acetate was proved to have taken part in the synthesis of lipoid since 14·7 % of the total lipoid hydrogen consisted of deuterium; the percentage of deuterium in the unsaponifiable material was 31·6. Since, in our experiments, incubation in an acetate solution approximately doubled the amount of lipoid originally present, and since the original lipoid did not contain deuterium, about 30 % of the newly formed lipoid hydrogen and 63 % of that of the newly formed unsaponifiable matter must have come from the trideuteracetate. Our determinations of the constituents of the lipoid formed after a brewery yeast had been incubated in Na acetate solutions, showed that the ratio of unsaponifiable to fatty acid was approximately 1 : 3, the same as in the original yeast before incubation.

If a similar relation holds in trideuteracetate solutions, approximately 20 % of the hydrogen of the newly formed fatty acid consisted of deuterium derived from the trideuteracetate. This is in agreement with our findings that the lipoid is formed by condensation of the acetate molecules.

The yeast carbohydrate (prepared by the method of Sevag & Cattaneo [1935] contained only 1·6 % of its total hydrogen as deuterium.

Although the calculation of the proportions of deuterium in the fatty acid and sterol synthesized from the trideuteracetate can only be a rough approximation, the very marked difference in the proportion of deuterated hydrogen in the unsaponifiable matter (63 %) and in the fatty acid (20 %) would seem to signify a different manner of condensation of the trideuteracetate. The comparatively small proportion of deuterium remaining in the fatty acid may signify that deuterated water is split off from the condensed molecules which would then be reduced to saturated chains by hydrogen atoms.

In the sterol synthesis, two out of three of its total hydrogen atoms must have been derived from the trideuteracetate.

The influence of phosphates on the formation of fat from acetate and from hexose

The mean lipoid content of yeast incubated in a 0·6 % solution of Na acetate was found to be unaffected by the addition of Na_2HPO_4 to the medium. Increasing the concentration of the acetate above 0·6 % diminished or failed to increase the fat content of the yeast and we could therefore only examine the influence of phosphate in dilute solutions. It is interesting that Katagiri [1926] found that

increasing the concentration of acetic acid in a glucose solution, buffered with Na acetate-acetic acid mixture, exercised an inhibitory effect on fermentation which could not be wholly ascribed to the *p*H.

When fat is formed from hexose, the effect of phosphate is much more obvious in concentrated than in dilute sugar solutions. Smedley-MacLean & Hoffert [1924, Table III] incubated yeast in oxygenated 4 % fructose solutions, the medium being changed every 48 hr.; their results show the close relationship that exists between the stored carbohydrate and lipoid when alkali phosphate is added to the fructose medium.

Consideration of the evidence at present available, seems perhaps to be most consistent with the view that when yeast is incubated in an oxygenated sugar solution, the formation of fat runs parallel with the storage of sugar and phosphate and is probably connected with some stage of the triosephosphate breakdown, pyruvic acid possibly providing the necessary starting material. This process is largely inhibited by the presence of Ca^{++} or Mg^{++} in the medium.

When fat is formed from acetate there is no indication that the hexosephosphate path is followed; there seems to be a mechanism by which acetate molecules can be directly condensed to form fatty acids; this process is also adversely affected by the presence of Ca^{++} or Mg^{++}. There seems to us to be no indication of the nature of the intermediate stages of this condensation. Wieland & Wille have suggested that acetic acid may be first converted into an "activated" form of succinic acid and that this may pass through oxaloacetic to pyruvic acid. This hypothesis though attractive lacks experimental basis.

Both in the formation of fat from hexose and from acetate, plentiful oxygenation is an essential condition.

Summary

1. When yeast was incubated in an oxygenated solution of any of the following substances, no increase of lipoid over that produced on incubation in oxygenated water was observed; acetoin, 2:3-butyleneglycol, methylethyl ketone, sodium salts of citric, succinic, maleic, fumaric, crotonic, laevulic or gluconic acids.

2. Solutions of substances containing two conjugated double bonds, inhibited the lipoid increase normally found on incubating in oxygenated water.

3. Addition of Ca^{++} or Mg^{++} to oxygenated glucose solutions in which yeast was incubated markedly diminished the amount of lipoid normally stored. Addition of Ca^{++}, Mg^{++} or Na^{+} to oxygenated glucose solutions in which yeast was incubated lowered the carbohydrate content whether added as chloride or acetate.

4. When yeast was incubated in oxygenated acetate solutions, Ca^{++} or Mg^{++} lowered the amount of lipoid normally formed from the acetate.

5. Addition of phosphate failed to increase the amount of lipoid formed from acetate when yeast was incubated in sodium acetate solutions (0·6 %).

6. Addition of K^{+}, Na^{+}, Ca^{++} or Mg^{++} to a glucose solution in which yeast was incubated, increased the amount of carbonyl substances present in the medium. In experiments in which the acetates of these metals were added to the medium, pyruvic acid and acetaldehyde were identified.

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CCVI. FACTORS INFLUENCING BACTERIAL DEAMINATION

III. ASPARTASE II: ITS OCCURRENCE IN AND EXTRACTION FROM *BACTERIUM COLI* AND ITS ACTIVATION BY ADENOSINE AND RELATED COMPOUNDS

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The bacterial deaminases of *Bact. coli*, previously studied, fall into two groups: the first consisting of the deaminases of glycine, *dl*-alanine and *l*-(+)glutamic acid, and the second of the deaminases of *dl*-serine and *l*-(−)aspartic acid. In these investigations deaminase activity has been measured in Q_N (μg. NH_3-N liberated per mg. dry wt. bacteria per hr.). The deaminases of the first group [Stephenson & Gale, 1937] carry out only oxidative deamination and have an activity of the order $Q_N = 30$ which remains constant throughout the growth period of the organism. Those of the second group [Gale & Stephenson, 1938] carry out both aerobic and anaerobic deaminations and have an activity of a much higher order—values of 1000 for *dl*-serine and 500 for *l*-(−)aspartic acid have been recorded—and this activity varies greatly with the age of the culture.

In the study of *dl*-serine deaminase, it was shown that the washed suspension of the organism in water lost activity on standing. Investigation of this loss and its subsequent recovery led us to postulate the existence of a diffusible coenzyme for serine deaminase. The loss of activity could be prevented by incubation with a reducing agent in the presence of phosphate, or with a trace of adenylic acid. Also, if the activity were allowed to fall, then the lost activity could be recovered by incubation for 1–2 hr. with a reducing agent and phosphate—neither being effective alone. From this evidence, we deduced that the coenzyme could exist in a reduced or oxidized form and in a phosphorylated or non-phosphorylated form, the reduced and phosphorylated form being the active substance.

Since this work was published, it has been demonstrated that the alanine oxidase preparation from kidney requires a coenzyme [Das, 1936; Warburg & Christian, 1938, 1; Straub, 1938] and that this coenzyme contains adenine, phosphate and flavin [Warburg & Christian, 1938, 2].

Harden [1901] showed that a culture of *Bact. coli* grown in glucose broth would deaminate aspartic acid with the formation of succinic acid. Quastel & Woolf [1926] showed that this reaction took place anaerobically in the absence of inhibitors but that in the presence of an inhibitor such as toluene, an equilibrium was set up corresponding to

$$\text{Aspartic acid} \rightleftharpoons \text{Fumaric acid} + NH_3,$$

the action of the toluene being to stop the later reduction of fumaric acid to succinic acid. Woolf [1929] showed that the fumarase of the organism produced malic acid from the fumaric acid so that the final equilibrium was really an

aspartic-fumaric-malic-NH_3 system. He gave the name "aspartase" to the enzyme responsible for the formation of fumaric acid from aspartic acid. These workers used anaerobic conditions for all their experiments and showed that the effect of toluene was to inhibit the reduction of fumaric acid but, since the evolution of NH_3 from aspartic acid in the initial stage of the experiment was independent of the presence of toluene, this inhibitor can have no action on the aspartase enzyme itself.

Part of this paper will be devoted to showing that there is, in *Bact. coli*, an enzyme, other than the "aspartase" of Woolf, which is responsible for the deamination of aspartic acid but which requires for its action the presence of a coenzyme which is destroyed by the method used by previous workers for the preparation of the bacterial suspension.

Methods. The strains of *Bact. coli* used, methods of growth, preparation and measurement of bacterial suspensions and the determination of NH_3 liberated have already been described [Gale & Stephenson, 1938]. Since the results obtained with the two strains do not differ significantly, only those obtained with strain I are presented here. Fig. 1 shows the effect of *p*H on deamination of *l*-(−)aspartic acid by this strain.

The adenosine used in part of this work was obtained from Dr George Henning of Berlin-Tempelhof.

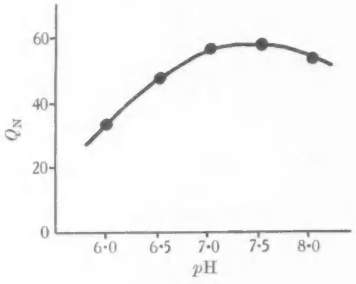


Fig. 1.

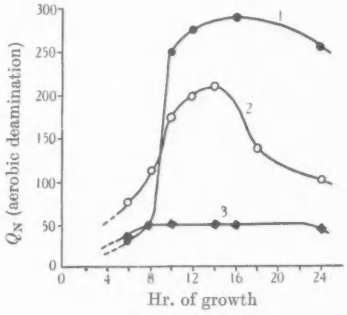


Fig. 2.

Fig. 1. Effect of *p*H on the rate of deamination of *l*-(−)aspartic acid by *Bact. coli* (I).

Fig. 2. Variation of deaminase activity with "age" of culture and conditions of growth: 1, in N_2; 2, in air; 3, in 2 % glucose broth in N_2 (strain I).

Variation of deaminase activity with "age" of culture and conditions of growth. Fig. 2 shows the Q_N variation due to culture time and conditions of aeration. In curve 1 the culture was grown anaerobically in N_2 for varying periods; in curve 2 the growth was carried out aerobically in liquid medium in Roux bottles; in curve 3 the organism was grown in 2 % glucose broth with chalk and bubbled continuously with N_2. In the last case samples were taken at intervals with sterile precautions, the chalk removed by centrifuging for 2 min. at 3000 r.p.m. and pouring off the supernatant liquid from which the organisms could then be removed by centrifuging in the usual way. The activities in each case were determined aerobically.

The presence of glucose in the growth medium inhibits deaminase activity, a result which has been shown with all the deaminases so far studied. The low activity obtained under such conditions remains constant with age. With curve 2,

however, there is a steadily increasing activity for the first 14 hr. of growth, after which the activity falls again, reaching the initial low value after about 24 hr. When the culture is grown anaerobically, there is a sharp rise in activity between the 8th and 10th hr. of growth and the high activity then attained does not decrease appreciably up to the 24th hr. of growth.

Fig. 3 shows how this variation of activity in an anaerobic culture is related to the growth curve and also to the E_h of the medium determined with the calomel electrode. The increase in activity reaches a maximum value at about the same time as active cell division ceases and the E_h reaches a steady value. It seemed likely that the increase in activity between the 8th and 10th hr. was due to a change in the medium consequent upon the metabolic activity of the cells. To investigate this point 900 ml. of liquid medium were inoculated with a 12 hr.

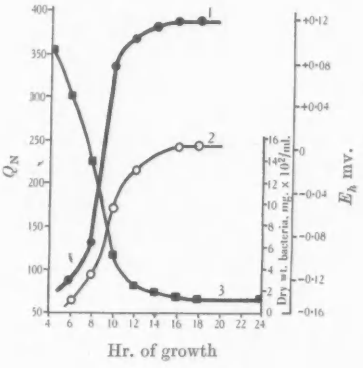


Fig. 3.

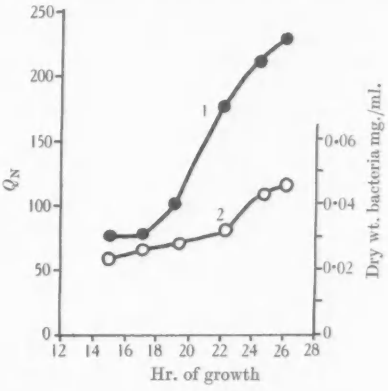


Fig. 4.

Fig. 3. 1, Variation of deaminase activity; 2, growth in dry wt.; 3, E_h of medium, with "age" of culture. Anaerobic culture (strain I).

Fig. 4. 1, Variation of deaminase activity; 2, growth curve for culture grown aerobically in inorganic medium + 2% lactate + 2% aspartate (strain I).

culture of the organism and incubated anaerobically for 10 hr., by which time the activity of the culture was approaching its maximum. The greater part of the organism was then centrifuged off and the medium sterilized by passage through a Seitz filter. This semi-exhausted medium was then re-inoculated with a 12 hr. culture, bubbled with N_2 and incubated. A turbidity was visible after 5 hr. and the organisms were spun off after 6 hr. The results of three determinations of the activity are set out in Table I. The earliest obtainable culture in the semi-

Table I. *Activity of early cultures grown anaerobically in "semi-exhausted" medium*

Exp.	Hr. of growth	Activity Q_N	Activity of control grown in normal medium (Q_N)
142	6	376	35
143	7	296	42
153	6	378	75

In exp. 153, 1% sodium aspartate was added to the semi-exhausted medium before the 2nd inoculation.

exhausted medium has an activity equal to the maximum activity attained by a culture grown normally for 14 hr. This indicates that variation in activity is dependent upon an alteration in the chemical constitution of the growth medium.

Fig. 4 shows that a culture grown in synthetic inorganic medium [Stephenson, 1930] with the addition of 2 % Na aspartate and 2 % Na lactate, shows an increase in activity before growth stops. If the increase in activity is due to the formation in the medium of some substance, then the organism is able to synthesize that substance from the constituents of this synthetic medium and, presumably, if that substance were added to the medium in the first place, then the earliest obtainable cultures would possess maximum activity. Accordingly 6 hr. cultures have been grown in liquid medium with the following additions and, in each case, the activity of the washed suspension determined:

1 % Na fumarate; 1 % Na succinate; 1 % Na pyruvate; 0·5 % Na formate; 0·5 % alanine; 0·05 % adenosine; excess CO_2.

In no case has a significant increase of activity over the control been obtained.

Loss and recovery of activity. The greater part of the work to be presented was carried out with cultures grown anaerobically for 15 hr. and having a Q_N (aerobic) of approximately 300 after preparation of the washed suspension. As in the case of serine deaminase, it was found that the activity of the washed suspension "decayed" on standing whether at 0° or on incubation. Table II shows that if suspensions of different strengths are incubated *in vacuo* for 3 hr. at 37° there is a loss of activity which increases with dilution of the suspension, being 28 % for a suspension of 3·7 mg./ml. and 78 % for a 0·37 mg./ml. suspension.

Table II. *Effect of dilution with water during incubation for 3 hr. at 37°*

Suspension of bacteria incubated (mg./ml.)	Dilution	Suspension of bacteria for estimation (mg./ml.)	Q_N (initial = 338)	Loss of activity %
3·70	—	0·37	244	28
0·75	1/5	0·37	177	48
0·37	1/10	0·37	75	78

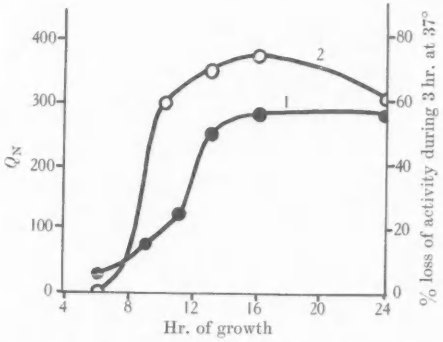


Fig. 5. Effect of "age" of (anaerobic) culture on: 1, activity (Q_N); 2, % loss of that activity during 3 hr. subsequent incubation at 37°.

With serine deaminase, it was found that the % loss of activity over any given period depended upon the "age" of the culture: an effect which was provisionally explained by differences in the permeability of the organism. Fig. 5 shows the relation between the "age" of the culture and its activity with aspartic acid (curve 1) and the % loss of activity over 3 hr. incubation *in vacuo* at 37° (curve 2). With serine deaminase, a maximum "permeability" was obtained between the 10th and 12th hr. of growth and the rate of loss of activity fell off rapidly on either side of this period: with aspartic deaminase, the rate of loss of activity remains approximately constant from the 12th to the 20th hr. of growth. The marked differences between the activity and the "permeability" curves for the two deaminases indicate that we are dealing with different systems in each case.

With serine deaminase, it was shown that the lost activity could be recovered again by incubating the bacterial suspension for 1–2 hr. with glutathione (GSH) or more negative reducing systems in the presence of phosphate. Similar experiments have been carried out with the aspartic deaminase and the results are set out in Fig. 6. A culture was grown anaerobically for 15 hr. and the aerobic activity of the washed suspension was determined immediately after its prepara-

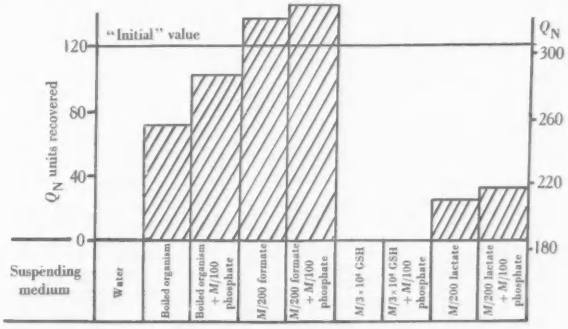


Fig. 6. Recovery of activity (strain I).

tion ("initial Q_N" = 306). The suspension was then left at 0° for 3 hr. *in vacuo*, when its activity had fallen to 186. The activity of the "decayed" suspension was then redetermined aerobically in the presence of borate buffer at *p*H 7·5 to which the following systems had been added: (1) water; (2) boiled bacteria in water; (3) boiled bacteria in 0·01 *M* phosphate buffer at *p*H 7·5; (4) formate, 0·005 *M*; (5) formate, 0·005 *M*, and phosphate, 0·01 *M*; (6) glutathione, *M*/30,000 GSH; (7) *M*/30,000 GSH and 0·01 *M* phosphate; (8) lactate 0·005 *M*; (9) 0·005 *M* lactate and 0·01 *M* phosphate (final concentrations). The results show the following marked differences from those obtained with similar experiments carried out with serine deaminase.

1. With certain additions an immediate recovery of activity is obtained without incubation.

2. GSH, with or without phosphate, has no effect either before or after incubation.

3. Boiled bacteria or formate produce an immediate reactivation with or without phosphate and, in certain cases, this reactivation proceeds to a point above the "initial" value.

Fig. 7 shows the effect of adding certain of these substances to the washed suspension immediately after its preparation. Boiled bacteria and certain reducing systems again produce an activation of the suspension. The preparation of the bacterial suspension takes about $1\frac{1}{2}$ hr. and it can be shown that there is a loss of activity during this period and that it is this lost activity which is recovered in Fig. 7. The reactivation obtained with lactate is not satisfactorily reproducible while that obtained with formate is consistent so that it seems that a reducing system is required which is rather more negative than the lactate-lactic dehydrogenase system.

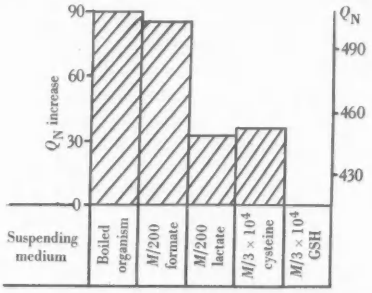


Fig. 7. Immediate activation produced by boiled organism or reducing systems.

The immediate reactivation effect of boiled bacteria suggests that they form a source of some essential factor which is lost on standing and a search for a coenzyme of *l*-(−)aspartic deaminase was next undertaken.

Nature of the coenzyme. A culture was grown anaerobically for 15 hr. and the initial aerobic activity of the washed suspension determined ($Q_N = 251$). After 3 hr. at 0° *in vacuo* its activity had fallen to $Q_N = 84$. The activity was then redetermined in the presence of (1) boiled cell-free liver extract; (2) boiled cell-free yeast extract; (3) a suspension of boiled bacteria; (4) a cozymase preparation in solution containing approximately 0·1 % coenzyme I; (5) a solution freshly prepared from a separate dry cozymase preparation containing 1 mg. of the dry material in 10 ml. The results are shown in Table III and a reactivation is obtained with the first four additions but not with the second of the cozymase preparations.

Table III. *Sources of coenzyme*

Initial activity of washed suspension	$Q_N = 251$
Activity after 3 hr. at 0° *in vacuo*	$Q_N = 84$

Addition to suspending medium	Resultant activity Q_N	Increase in Q_N
Boiled liver ext.	227	143
Boiled yeast ext.	161	77
Boiled bacteria	229	148
Cozymase prep. A	202	118
Cozymase prep. B	88	4

To confirm that the deaminase factor was not coenzyme I, the cozymase preparation which had proved active in the last experiment was treated in the various ways set out in Table IV. Coenzyme I is destroyed by boiling at *p*H 12 for 2 min. but is stable to boiling at *p*H 1 for 5–10 min. If, however, the

coenzyme is first reduced with $Na_2S_2O_4$ then these relations are reversed, the reduced form being stable to alkali and unstable to acid [Green & Brosteaux, 1936]. Excess $Na_2S_2O_4$ was oxidized by aeration. The form of the experiment was similar to those already described and it is obvious that the deaminase factor bears no relation to the activity of coenzyme I but would appear to increase slightly during alkaline hydrolysis and to decrease during acid hydrolysis. The factor is then, presumably, either an impurity in the cozymase preparation or a breakdown product of coenzyme I. The known impurities in the active cozymase preparation were then tested and are also listed in Table IV where it is seen that adenylic acid possesses a definite reactivation effect but of a rather low order.

Table IV. *Nature of active factor in cozymase prep. A*

Initial activity of washed suspension $Q_N = 292$
Activity after 3 hr. at 0° *in vacuo* $Q_N = 206$

Addition to suspending medium	Previous treatment. Boiled for 5 min. at *p*H	Coenzyme I	Resultant activity Q_N	Q_N increase	Factor
Cozymase prep. A	—	Active	297	91	+
,,	12	Inactive	324	118	+
,,	1	Active	304	98	+
Cozymase reduced	12	Active	277	71	+
,,	1	Inactive	266	60	+
Cozymase prep. B	—	Active	200	—	—
Flavine	—	—	196	—	—
Adenylic acid (0·1 mg./ml.)	—	—	246	40	+
Nicotinamide (0·1 mg./ml.)	—	—	205	—	—
Boiled bacteria	—	—	290	84	+

It has been shown that adenine compounds are readily attacked by *Bact. coli*, the probable course being as follows [Lutwak-Mann, 1936]:

Adenylic acid → adenosine → inosine → hypoxanthine + ribose
Inosinic acid → inosine → hypoxanthine + ribose

For this reason adenylic acid and its decomposition products were tested for their activating effect as follows: the activity of a washed suspension from a 15 hr. anaerobic culture was determined aerobically immediately after its preparation in the usual way with 0·03 *M* phosphate buffer and *M*/250 *l*-aspartate and then also in the presence of (1) adenylic acid; (2) inosinic acid; (3) adenosine; (4) inosine; (5) adenine; (6) hypoxanthine; (7) ribose; (8) boiled bacteria. The final concentration in all cases, with the exception of (8), was 1 mg./100 ml. The results are shown in the top portion of Fig. 8 as "initial recovery". The suspension was then allowed to stand in water at 0° for 3 hr. by which time its activity had fallen to $Q_N = 100$ and the whole experiment then repeated with this "decayed" suspension: the results obtained are shown in the lower portion and overlapping the upper portion of Fig. 8. The most effective substance is adenosine, having an activation effect greater than that produced by the boiled bacteria. It is closely followed by inosine and inosinic acid. The further breakdown products of adenosine—adenine, hypoxanthine and ribose—produce a small reactivation in the second half of the experiment but their effect is generally rather less than in the example quoted.

These results suggest that adenosine is the activating substance replaceable by inosine or inosinic acid, possibly in virtue of their interchangeability under the action of the organism [Stephenson & Trim, unpublished]. The lower but still

marked activity of the other decomposition products of adenosine may be due to the synthesis from them of one of the three more active compounds. Until the mode of action of the adenosine etc. is explained, speculation on this point is useless. At first sight it might be thought that adenosine and inosine act as an

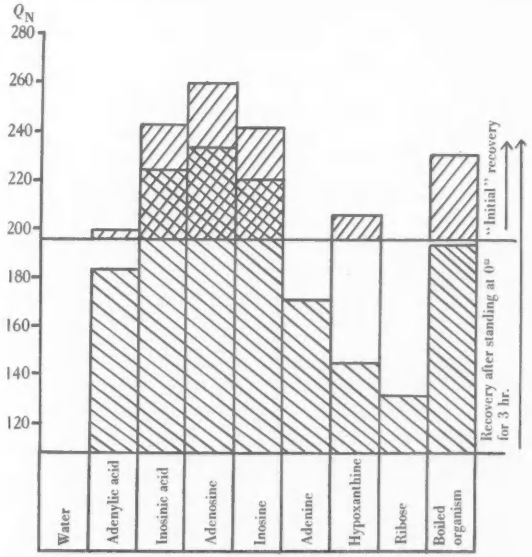


Fig. 8. Recovery in the presence of breakdown products of adenylic acid.

NH_3-carrier system, the inosine reacting with aspartic acid to form adenosine and the latter then being deaminated to inosine again. That this cannot be the case is seen from a consideration of the rates of deamination of aspartic acid and adenosine respectively:

Rate of deamination of *l*-aspartic acid:

(*a*) Aerobically in presence of adenosine $Q_N = 450$
(*b*) Anaerobically in presence of adenosine $Q_N = 501$

Rate of deamination of adenosine:

(*a*) Aerobically $Q_N = 122$
(*b*) Anaerobically $Q_N = 120$

The effect of adenosine on the deamination of l-(−)*aspartic acid.* It was next necessary to determine whether this co-deaminase effect of adenosine would work anaerobically.

Fig. 9 shows the effect of adding an initial concentration of 0·67 mg. adenosine/ml. on the deamination and Fig. 10 the corresponding effects with one-quarter this quantity of adenosine. In the absence of adenosine there is a much slower rate of evolution of NH_3 anaerobically than aerobically. The effect of the addition of the adenosine is to increase the rate both aerobically and anaerobically but, whereas the aerobic rate is increased by a constant anaerobically the initial acceleration is much greater but rapidly falls off until the rate is back

to that of the control. That this is not due to the attainment of an equilibrium can be demonstrated by the addition of further adenosine, when the rate will again be temporarily accelerated.

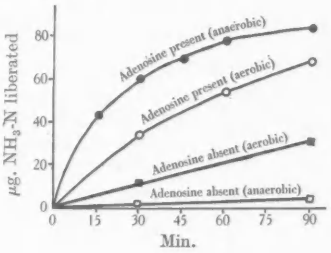


Fig. 9.

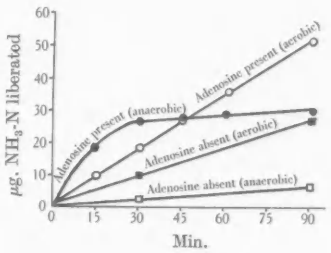


Fig. 10.

Fig. 9. Effect of adenosine (1/15 mg./ml.) on evolution of NH_3 from *l*-(−)aspartic acid in presence of *Bact. coli* (0·1 mg. dry wt./ml.).

Fig. 10. Effect of smaller concentration of adenosine (1/60 mg./ml.) on evolution of NH_3 (cf. Fig. 9).

Fig. 11 shows the result of adding increasing concentrations of adenosine upon the Q_N. Since the accelerated rate is constant aerobically (Figs. 9 and 10) we can determine a true rate under such conditions but anaerobically this is not possible and the points on the anaerobic curve of Fig. 11 represent the rate for the first 30 min. after the addition of the adenosine. We have seen from Figs. 9 and 10 that a given amount of adenosine produces a more marked increase in the anaerobic than the aerobic rate and this may mean that the total activity obtained aerobically is due to an anaerobic process. In the absence of adenosine and with freshly prepared suspensions, the aerobic rate of deamination is greater than the anaerobic but this difference in the two rates disappears on incubation of the suspension for half-an-hour. Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and this difference in anaerobic and aerobic activities of fresh cultures may be due either to a labile oxidative deaminase or to the natural adenosine of the bacterium having an effect which is obvious aerobically but falls off so rapidly anaerobically (cf. Fig 9) as not to be demonstrable.

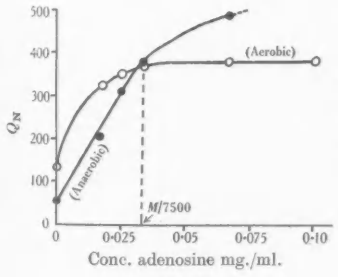


Fig. 11.

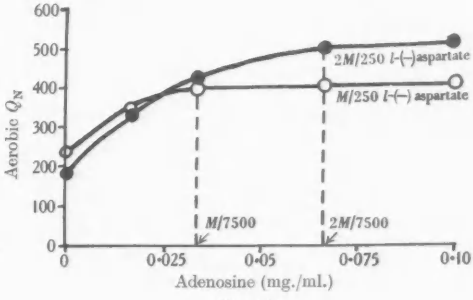


Fig. 12.

Fig. 11. Effect of adenosine concentration on rate of deamination by strain I.

Fig. 12. Variation of critical concentration of adenosine with concentration of *l*-(−)aspartate.

From Fig. 11 it is also seen that, aerobically, the rate of deamination reaches a maximum in the presence of a final concentration of adenosine greater than the critical concentration of $M/7500$. The final concentration of aspartic acid in this case is $M/250$ so that a maximum rate of deamination is obtained when there is one mol. adenosine present to every 30 mol. aspartic acid. Fig. 12 shows that if the final concentration of *l*-(−)aspartic acid is doubled then the critical concentration of adenosine is also approximately doubled.

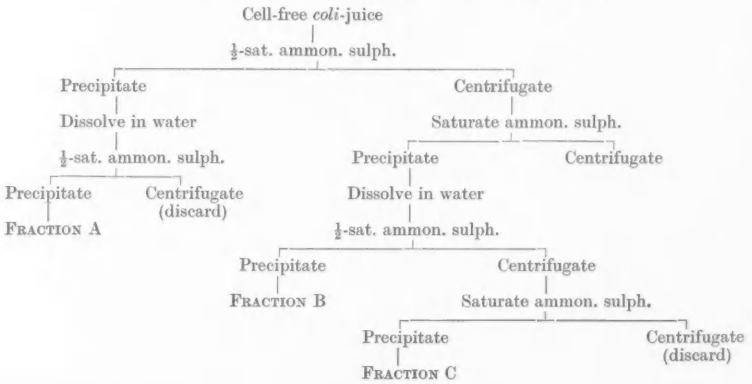
Deamination of l-(−)*aspartic acid by cell-free* coli-*juice.* At this point in the research the wet-crushing mill of Booth & Green [1938] became available and it was decided to test these findings with a cell-free juice obtained by crushing *Bact. coli*. 25 Roux bottles containing broth-agar with the addition of 0·5 % Na lactate were sown with the organism and incubated for 24 hr., the culture then washed off and centrifuged, washed once in water and then made up into a thick cream (*c.* 35 ml.). This was poured into the mill and ground for 2 hr. by which time most of the cells had been disintegrated. The resulting juice was then centrifuged until microscopic examination showed it to be free from whole cells.

The juice was then tested for activity as aspartic deaminase and the results obtained are shown in Table V. 1 ml. each of the juice, $3M/250$ *l*-(−)aspartate and $M/10$ phosphate buffer at *p*H 7·5 were shaken aerobically in a bath at 40°. The figures given are corrected for the blank of the juice. The activity was then redetermined in the presence of adenosine and also after dialysis with and without adenosine. It appears that neither dialysis nor the addition of adenosine has any appreciable effect on the activity. This may be due to the fact that the juice contains the optimal activating amount of adenosine already. Also, since

Table V. *Deamination of* l-*aspartate by cell-free* coli-*juice*

	μg. NH_3-N liberated/ hr./ml. juice
(*a*) Juice + $M/250$ aspartate	97
(*b*) Juice + $M/250$ aspartate + 0·07 mg. adenosine	126
(*c*) Juice + 0·05 mg. adenosine	18
(*b*)–(*c*)	108
(*d*) Dialysed juice + $M/250$ aspartate	192
(*e*) Dialysed juice + $M/250$ aspartate + 0·07 mg. adenosine	107
(*f*) Dialysed juice + 0·07 mg. adenosine	18
(*e*)–(*f*)	89

Table VI. *Scheme of fractionation of* coli-*juice*



the bacteria had been grown in a manner different from that used for the organisms used in the first part of the work, it was possible that the juice contained other enzymes capable of deaminating aspartic acid.

Hence an attempt was made to fractionate the juice (Table VI).

Separation of aspartase I and aspartase II from cell-free coli-*juice*. The scheme provides three precipitates. Each of these was dissolved in water and then dialysed against distilled water until a blank of 10–20 μg. NH_3-N/ml. of preparation, was obtained. Each of the preparations was then tested for deaminase activity with *l*-(−)aspartic acid, the activity redetermined in the presence of adenosine at a final concentration of 1/10 mg./ml. and, finally, the preparation was incubated with toluene for 2 hr. and its activity then determined in the presence of adenosine (1/10 mg./ml.). The results of one such set of experiments is given in Table VII where the activities are expressed as μg. NH_3-N liberated per hr. per 100 mg. dry wt. of preparation. Fraction A has a small blank activity which is increased some 500% by the presence of adenosine and its activity is completely abolished by incubation with toluene; fraction B appears to have no activity towards *l*-aspartic acid under any of the circumstances tested. Fraction C has an activity which is unaffected by added adenosine and is completely unaltered by toluene treatment. These experiments were carried out anaerobically. The course of deamination by fraction A under various conditions is set out more fully in Fig. 13.

Table VII. *Analysis of fractions from* coli-*juice*

	μg. NH_3-N/hr./100 mg. dry wt.		
	Fraction A	Fraction B	Fraction C
(*a*) *M*/250 aspartate	110	0	90
(*b*) *M*/250 aspartate + 0·1 mg. adenosine	650	0	75
(*c*) 0·1 mg. adenosine	70	0	0
(*b*)–(*c*)	580	0	75
(*d*) Fraction (after 2 hr. with toluene at 37°) + *M*/250 aspartate + 0·1 mg. adenosine	0	0	85

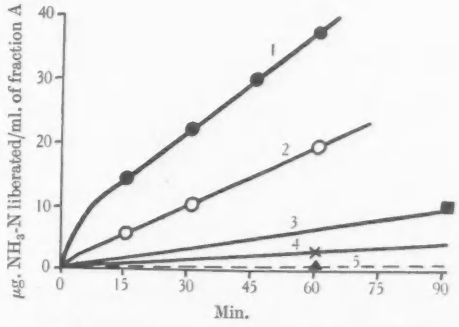


Fig. 13. Deamination of *M*/250 aspartate by fraction A: 1, + adenosine 0·1 mg./ml.; 2, + adenosine 0·03 mg./ml.; 3, no addition; 4, adenosine alone 0·1 mg./ml.; 5, same as 1, with toluene-treated fraction.

This fractionation leads to the separation of two distinct enzyme systems which deaminate *l*-(−)aspartic acid anaerobically. The enzyme of fraction C is unaffected by toluene or adenosine and must therefore be the one studied by

previous workers and called "aspartase" by Woolf [1929]. It is proposed that, in future, this enzyme should be called "aspartase I" and the enzyme present in fraction A whose activity depends upon the presence of adenosine and is completely inhibited by toluene treatment, "aspartase II". The enzyme separated from *Bact. fluoresc. liquefaciens* [Virtanen & Tarnanen, 1932] was probably, since toluene was used in the extraction, aspartase I.

Distribution of aspartase I and II in Bact. coli. It has been possible to demonstrate the presence of aspartase II in cultures of *Bact. coli* of both strains used in this laboratory and grown in the various conditions set out in Table VIII. Toluene treatment of the bacterial suspension completely inhibits aspartase II but, according to the work of Quastel & Woolf [1926], has no effect on aspartase I. Hence the Q_N determined after such treatment and measured during the initial stages of the deamination while the evolution of NH_3 is linear, gives a measure of aspartase I activity. From Fig. 11 we see that the rate of deamination is constant aerobically in the presence of a concentration of adenosine greater than the critical value and we can take this rate as a measure of the total activity. Assuming for the present that this total activity is the sum of the activities of aspartase I and II, we obtain that of aspartase II by difference. All the relevant figures are set out in Table VIII. The ratio of aspartase II/aspartase I shows that quantitatively the aspartase II is the more important enzyme in all cases. With strain I, aspartase I is negligible in the anaerobic cultures and it is with this strain that the greater part of the work has been carried out. Strain II shows approximately the same total activities for each type of culture as strain I but contains, in every case, a higher degree of activity due to aspartase I so that the ratios are of a lower order throughout.

Table VIII. *Analysis of deaminase activity and distribution of aspartase I and II in* Bact. coli

	Aerobic deamination Q_N				Anaerobic deamination Q_N						
			Toluene treated								
Adenosine	−	+	−	+	−	+	Tol. treat. +	Total activity	Aspartase I	Aspartase II	Ratio II/I
Conditions of growth											
Strain I:											
Anaerobic in broth	138	364	8	7	32	401	7	364	7	357	51
Broth in flask	150	250	23	6	32	114	4	250	4	246	62
Aerobic in broth	166	331	28	30	27	348	50	331	50	281	4
Aerobic on agar	96	121	6	6	17	127	9	124	9	115	13
2% glucose broth	34	75	12	15	26	83	20	75	20	55	3
Strain II:											
Anaerobic in broth	75	449	36	36	77	395	80	449	80	369	4·6
Aerobic in broth	312	437	70	48	114	400	60	437	60	377	6
2% glucose broth	54	95	32	32	40	106	32	95	32	63	2

It can now be seen why previous workers should have overlooked the existence of aspartase II. Woolf [1929] grew his cultures in broth in Roux bottles and so would have obtained the highest content of aspartase I. Growth was allowed to continue for 48 hr. by which time, as can be seen from Fig. 2, the total activity would have fallen to a low figure. The organisms were washed 3 times and the suspensions then aerated for 1 hr. so that a considerable "decay" of aspartase II activity would occur. The suspension was kept for use up to a fortnight and it is stated that the activity remained constant for 2 months whereas aspartase II

activity decays to zero within 12–24 hr. Finally, the actual experiments were carried out anaerobically when the small amounts of adenosine present would have no significant effect and also after toluene treatment which inhibits the action of aspartase II.

Effect of formate on deamination of l-*aspartic acid by* Bact. coli. In the early experiments of this series, a reactivation of a "decayed" suspension was obtained with boiled bacteria or with formate. The action of the boiled bacteria can now be explained since Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and is a plentiful source of its precursors. The effect of formate remains to be investigated.

Figs. 14 and 15 show the effects on the anaerobic and aerobic deaminations of the addition of the critical amount of adenosine (1), *M*/120 formate (2), and of the two together (3). Aerobically, the formate has the same accelerating effect as adenosine but anaerobically, after a brief marked acceleration of the rate, the effect of formate is scarcely significant.

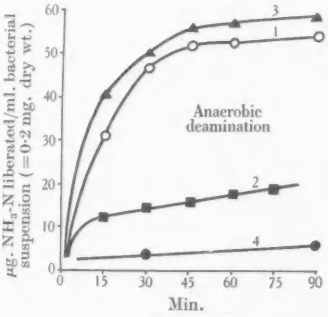


Fig. 14.

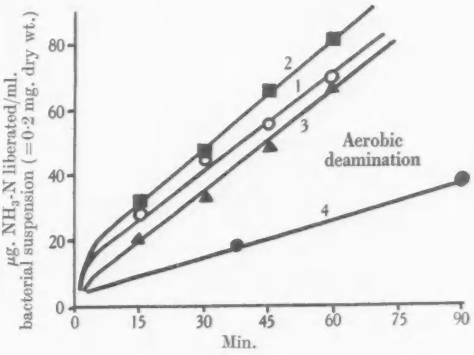


Fig. 15.

Fig. 14. Anaerobic liberation of NH_3 from *l*-(−)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0·03 mg./ml.; 2, *M*/120 formate; 3, *M*/120 formate + adenosine 0·03 mg./ml.; 4, alone.

Fig. 15. Aerobic liberation of NH_3 from *l*-(−)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0·03 mg./ml.; 2, *M*/120 formate; 3, *M*/120 formate + adenosine 0·03 mg./ml.; 4, alone.

Fig. 16 shows the effect of increasing formate concentration on the aerobic Q_N. For comparison the effects of similar concentrations of adenosine are marked with a dotted line and it is seen that, to attain a maximum rate, a much higher concentration of formate than of adenosine is required but that the maximum rate reached is the same in both cases. With strain I, the critical concentration of formate would appear to be slightly greater than *M*/1500.

Fig. 17 shows the results obtained in an experiment with the aspartase II fraction of *coli*-juice in which the rate of deamination measured in μg. NH_3-N liberated per 30 min. per ml. of preparation is determined in the presence of various concentrations of adenosine (1). The deamination is followed aerobically and a maximum rate is obtained with a concentration of 0·125 mg./ml. adenosine. The experiment was then repeated in the presence of *M*/6000 formate (2) with the result that the maximum rate of deamination was reached in the presence of 0·0625 mg./ml. adenosine, so that the critical concentration of adenosine is halved by the presence of the formate. In the absence of adenosine, formate alone has no consistent or appreciable effect on deamination by the fraction.

Fig. 18 shows the results of an experiment similar to the last, carried out on a suspension of the bacteria. The critical concentration of adenosine for maximum rate of deamination was determined and gave the usual value of 1/30 mg./ml. The experiment was then repeated in the presence of $M/6000$ formate and again

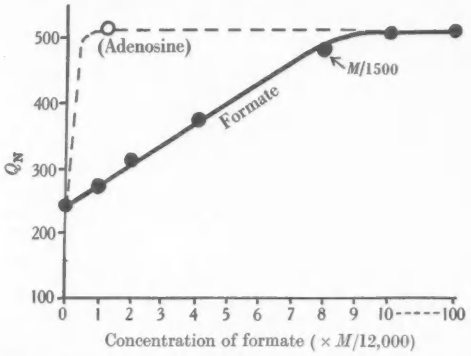


Fig. 16. Effect of formate (and adenosine) on aerobic deamination.

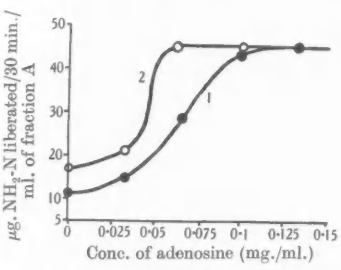


Fig. 17.

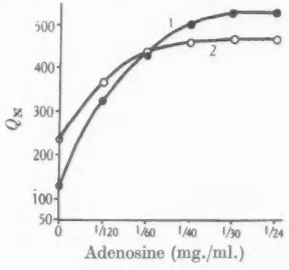


Fig. 18.

Fig. 17. Rate of deamination of *l*-(−)aspartate by fraction A of *coli*-juice in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

Fig. 18. Rate of deamination of *l*-(−)aspartate by *Bact. coli* in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

the optimal concentration of adenosine was approximately halved by this concentration of formate. Reference to Fig. 16 shows that $M/6000$ formate is much less than the critical concentration of formate required by strain I to give the maximum Q_N.

Since the presence of $M/6000$ formate halves the amount of adenosine that must be added to give a maximum Q_N, and $M/1500$ formate will give this maximum Q_N in the absence of added adenosine while the organism already contains a certain amount of adenosine, it is suggested that the presence of sufficient formate decreases the amount of adenosine required until the system is maximally activated by the natural adenosine of the organism.

Products of the action of aspartase II on l-(−)*aspartic acid.* A preparation of aspartase II was made according to the scheme set out above and the fraction dissolved in water and dialysed overnight. In this way 40 ml. of a solution of

aspartase II were obtained. This was set up in a large Thunberg tube with 25 ml. Na aspartate equiv. to 1 g. *l*-(−)aspartic acid; 50 mg. adenosine dissolved in 15 ml. water, and 20 ml. *M*/5 phosphate buffer at *p*H 7·5. The tube was evacuated and placed in a bath at 40°; 0·2 ml. of the contents was withdrawn every 30 min. and the NH_3-*N* determined to follow the course of the reaction. After 5 hr. when the deamination had almost stopped at *c.* 50 % completion the reaction was stopped by acidifying with H_2SO_4.

In a preliminary experiment, acid was added to a concentration of 10 % and the solution extracted with ether in a separating funnel. The ether extract was evaporated to dryness, the residue taken up in water and precipitated with conc. $AgNO_3$. The white precipitate obtained was filtered off, resuspended in water and decomposed with H_2S, the Ag_2S filtered off and the clear filtrate evaporated to small bulk. On standing about 20 mg. of colourless crystals were obtained. A portion of these, dissolved in water, gave a white precipitate with $AgNO_3$, decolorized $KMnO_4$ and Br water and gave the reactions typical of fumaric acid. M.P. (dec.) 200° in open tube with sublimation, M.P. 240° in sealed tube, which is considerably lower than the M.P. (287°) of pure fumaric acid. Thus the product was impure and represented a very low yield.

Accordingly the exp. was repeated. After 5 hr. incubation the reaction was stopped by acidifying slightly with H_2SO_4 and boiling to precipitate the protein which was then filtered off. The filtrate was neutralized and evaporated to 15 ml. and then extracted continuously with ether in a liquid extractor for 6 hr. The ether extract was then evaporated to dryness and the residue dissolved in 25 ml. of water.

The water solution was analysed for fumaric, succinic and malic acids according to the method of Needham [1927]. 1 ml. of the solution was neutralized with sat. $Ba(OH)_2$ to remove sulphate and phosphate; the precipitate was filtered off immediately and washed with water.

97 % alcohol was then added to a final concentration of 36 % and the *p*H adjusted to 7·0. 2 ml. conc. $AgNO_3$ were then added and the precipitate was filtered off on a Gooch crucible. After thorough washing with 60 % alcohol, the precipitate was redissolved in dil. H_2SO_4 and the total Ag estimated by titration in the cold against 0·01 *N* KCNS using iron alum as external indicator. The AgSCN was filtered off and the filtrate analysed for the above acids.

Malic acid was estimated by the method of Auerbach & Kruger [1923] and it was shown that, under the conditions of the experiment, fumaric acid could be estimated by titration at 40° in the presence of H_2SO_4 against 0·01 *N* $KMnO_4$ (1 ml. 0·1 *N* $KMnO_4$ = 1·27 mg. fumaric acid).

Table IX. *Analysis of residue from ether extraction*

Residue dissolved in 25 ml. water		
Total Ag obtained from 1 ml.	= 11·8 mg.	
Fumaric acid/ml. estimated against *N*/100 $KMnO_4$	= 2·47 mg. ≡ 4·7 mg. Ag	
Malic acid/ml. estimated polarimetrically	= 4·1 mg. ≡ 6·6 mg. Ag	
Total silver/ml. due to known acids	= 11·3 mg.	
Total malic acid in residue	= 102·5 mg. ≡ 102 mg. aspartic acid	
Total fumaric acid in residue	= 61·8 mg. ≡ 70 mg. aspartic acid	
Total aspartic acid equivalence of residue	= 172 mg.	49 % recovery
Total aspartic acid disappeared (from NH_3 determinations)	= 348 mg.	

From Table IX it can be seen that the total acids of the extract as determined by the Ag salts can be accounted for as malic and fumaric acids. However, the total yield of these acids will account for only 50 % of the aspartic acid that has

disappeared but Needham [1927] showed that malic acid cannot be extracted quantitatively from water solution owing to its unfavourable partition coefficient. Since it would appear that only malic and fumaric acids are produced, these can both be estimated accurately in the actual reaction mixture if this is first deproteinized and evaporated to a small bulk. Table X gives the results of such an experiment and it can be seen that the aspartic acid disappearing is satisfactorily accounted for by the malic and fumaric acids appearing.

Table X. *Products of aspartase II action*

Total aspartic acid disappeared	=285 mg.
Total malic acid formed	=162·5 mg. ≡162 mg. aspartic acid
Total fumaric acid formed	=116·0 mg. ≡133 mg. aspartic acid
Equivalent total aspartic acid	=295 mg.

Thus the action of the fraction A obtained from *coli*-juice upon *l*-(−)aspartic acid is to produce NH_3 together with fumaric and malic acids. In the extract, the sum of the fumaric and malic acids does not account for the aspartic acid disappearing, but if these are estimated in the reaction mixture itself, then complete equivalence is obtained.

The presence of fumarase in fraction A. The formation of both malic and fumaric acids in the last experiment suggests that the fraction A obtained from *coli*-juice contains fumarase in addition to aspartase II. This was shown to be the case by the following experiment.

40 ml. of a preparation of fraction A were incubated *in vacuo* with 20 ml. phosphate buffer at *p*H 7·5 and 500 mg. fumaric acid made up into 20 ml. of neutral solution. At intervals of ½, 1, 2, 4, 6 and 24 hr. a 5 ml. sample was withdrawn and added to 10 ml. of 14·2 % ammonium molybdate solution. A similar sample was added to 11 ml. of 6 % trichloroacetic acid. 1 ml. of glacial acetic acid was then added to the first mixture and both samples were left in the dark for 3–4 hr. The protein was precipitated by both sets of reagents and was filtered off before the final estimation. The filtrate was made up in each case to 22 ml. for convenience and the malic acid estimated by the difference in rotation of the two samples [Auerbach & Kruger, 1923].

Table XI shows the formation of malic acid from fumaric acid in this experiment and thus demonstrates the presence of fumarase in the fraction.

Table XI. *Presence of fumarase in fraction A of* coli-*juice*

Initial concentration of fumaric acid = 6·25 mg./ml.

Increase in rotation (Hg green) in presence of molybdic acid due to 10 mg. malic acid under experimental conditions = +0·80°.

Time	Increase in rotation in presence of molybdic acid	Malic acid mg./5 ml. sample	Malic acid mg./ml.
0	0	0	0
30 min.	+0·47°	5·25	1·05
1 hr.	+0·87	10·9	2·2
2 hr.	+1·35	16·9	3·4
4 hr.	+2·09	26·1	5·2
6 hr.	+2·11	26·4	5·3

Summary

1. The deaminating activity of *Bact. coli* towards *l*-(−)aspartic acid varies (*a*) with the conditions of growth of the organism, and (*b*) with the age of the culture. The latter variation can be shown to be due to an alteration in the

chemical constitution of the growth medium consequent upon the metabolic activity of the cells.

2. Washed suspensions lose their activity upon standing and the rate of loss depends upon the dilution of the suspension.

3. The lost activity can be immediately recovered by the addition of boiled bacteria or formate to the suspension.

4. The recovery obtained with boiled bacteria resembles a coenzyme effect.

5. The coenzyme can be replaced by adenylic acid and its breakdown products, the most active being adenosine.

6. The addition of adenosine to the bacterial suspension results in a greatly increased rate of deamination aerobically or anaerobically.

7. A cell-free juice obtained by crushing and centrifuging *Bact. coli* will deaminate *l*-(−)aspartic acid.

8. A scheme for fractionation of the juice with $(NH_4)_2SO_4$ is given: this scheme results in the separation of two enzymes capable of deaminating *l*-(−)aspartic acid anaerobically. These enzymes are provisionally named:

Aspartase I which is unaffected by toluene and/or adenosine.

Aspartase II which is completely inhibited by toluene treatment, requires the presence of a coenzyme for its action, and is activated *in vitro* by adenosine.

9. The distribution of aspartase I and II in cultures of *Bact. coli* (two strains) grown under varying conditions has been determined.

10. Formate produces an activation of aspartase II aerobically but not significantly anaerobically.

11. Formate appears to reduce the amount of adenosine required to activate aspartase II.

12. The action of the aspartase II-fraction of *coli*-juice on *l*-(−)aspartic acid is to produce NH_3 and fumaric and malic acids.

13. The aspartase II-fraction of *coli*-juice also contains fumarase.

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CCVII. THE PRODUCTION OF INDOLE BY SUSPENSIONS OF *BACT. COLI*

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(*Received 23 July 1938*)

Woods [1935, 1] has studied the conversion of tryptophan into indole by washed suspensions of *Bact. coli*. He found that the process was an oxidation requiring 5 atoms O per mol. tryptophan. The conversion of tryptophan to indole was complete and the rate of disappearance of tryptophan was the same as the rate of formation of indole. The coli suspensions used by Woods were prepared from growths on trypsin-digested casein solidified with agar, and thus had always been produced in the presence of tryptophan. Happold & Hoyle [1935] also studied the oxidation of tryptophan and found on two out of three occasions that slight activity was present in suspensions grown in the supposed absence of tryptophan on Fraenkel's synthetic medium. Since this medium, however, contained natural asparagine [Dr Happold, private communication] it is possible that tryptophan might have been present as an impurity in biologically significant amounts. It was therefore decided to test the production of the enzyme under conditions in which tryptophan was certainly absent.

Technique

(1) *Medium used for growth of the suspensions.*

KH_2PO_4	...	...	4·5 g.
$(NH_4)_2SO_4$	...	...	0·5 g.
NH_4Cl ...	...	...	0·5 g.
M/2 Na lactate		...	50 ml.
N NaOH	...	...	26 ml.
Water to	...	...	950 ml.

This mixture was adjusted to *p*H 7·6, distributed in 9·5 ml. volumes in tubes and autoclaved.

The following solutions were prepared separately: $MgSO_4$, $7H_2O$, 0·4% (autoclaved); ferrous ammonium sulphate *M*/500 in *N*/50 HCl (filtered); Na dithiodiacetate *M*/200 (filtered).

These were mixed in the proportions Mg 1·0, Fe 2·5, dithioacetate 2·0, and 0·5 ml. added per tube. The dithiodiacetic acid was prepared as stated by Fildes & Richardson [1937]. Na lactate was prepared from three times recrystallized Zn lactate.

The mixtures, having been prepared in the test tubes and inoculated were poured into 50 ml. Erlenmeyer flasks and incubated at 37° in air + 5% CO_2.

All apparatus was treated in the usual way for excluding the presence of unknown substances.

(2) *The strains used.* Two laboratory cultures maintained on agar and four recent isolations were used. These gave the fermentation reactions of *Bact. coli*, *Bact. coli communis* and *Bact. acidi lactici*. All grew copiously after little hesitation on the medium described and in serial subculture from one drop of the previous culture in 10 ml. All produced indole rapidly when tryptophan

was added to the culture medium. Though some of the observations to be described were checked on more than one strain, a single strain was used as the standard. This was obtained from the cultures of the Bland-Sutton Institute and was described as No. 86 of the National Collection of Type Cultures. After 20–24 hr. growth the cultures were centrifuged and the supernatant liquid discarded. The bacteria were washed once with distilled water and suspended to a standard opacity in $M/18$ phosphate buffer (pH 7·6). The dry weight of bacteria from 1 ml. of this suspension was 1·5 mg.

(3) *Estimation of activity of the suspensions.* The bacterial suspension (7 ml.) was mixed in a 100 ml. Erlenmeyer flask with $M/18$ phosphate at pH 7·6 (12·5 ml.) and 0·5 ml. 0·175 % tryptophan, and incubated at 37°. The quantity of tryptophan used represented a possible maximum yield of indole of 25 μg./ml. Woods used four times this concentration.

The indole produced in the mixture was estimated by a simple method which appeared to be sufficiently accurate and gave results comparable with those of Woods.

A standard solution of indole was prepared containing 20 μg./ml. and from this a series of 10 dilutions with 25 % differences. One ml. of each dilution was mixed with 1 ml. of Ehrlich's indole reagent, prepared freshly from a sample of p-dimethylaminobenzaldehyde as colourless as possible. This reference series was remade after 4 hr.

The suspensions to be tested were centrifuged and the supernatant liquid diluted with water. One ml. of each dilution was mixed with 1 ml. Ehrlich reagent and after a few minutes tested against the reference series. The average of the estimates obtained from three or four dilutions was taken as the correct reading.

Various indole derivative tests by this technique at concentrations equivalent to 20 μg. indole/ml. gave negative results, namely indoleacrylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid. Indolecarboxylic acid gave a mere trace of colour which was due to indole which the preparation contained.

The acitvity of suspensions of bacteria grown without tryptophan

Each of the six strains grown under the conditions stated actively converted tryptophan into indole. Fig. 1 shows examples of the action of different suspensions of one strain (No. 86, N.C.T.C.) grown on different occasions. No. 86 was equally active after 40 subcultures since its last contact with tryptophan.

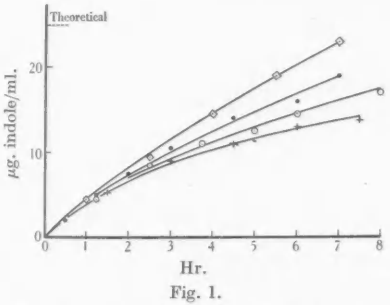

Fig. 1.

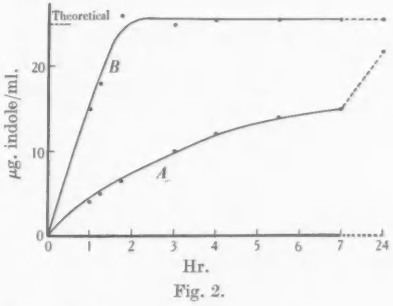

Fig. 2.

Fig. 1. Rate of production of indole by suspensions grown without tryptophan.

Fig. 2. Rate of production of indole by suspension *A* grown without tryptophan, compared with *B* grown with tryptophan.

The effect of growth in the presence of tryptophan

Parallel cultures were put up, one containing 0·5 ml. 0·175 % tryptophan per 10 ml. The suspensions were gathered after 22 hr. and gave the results shown in Fig. 2. The presence of the tryptophan did not increase the yield of the culture. Fig. 2 shows that growth in the presence of tryptophan much increased the activity of the suspension.

That the effect of growth in tryptophan is quantitative rather than qualitative is shown in Fig. 3. The suspension from a tryptophan culture was diluted serially with buffer until the initial activity was equal to that of the suspension grown without tryptophan. The course of the curves was exactly the same. In this experiment it was found that the activity of two suspensions could not be estimated from the initial courses of the curves. According to these it was assumed that the tryptophan culture was about five times more potent whereas it had to be diluted twenty-five times before its activity was the same as that of the culture grown without tryptophan.

These results obtained when tryptophan was present in the culture were in general confirmation of those of Woods and indicated that the less elaborate method used for estimating indole did not lead to any notable error.

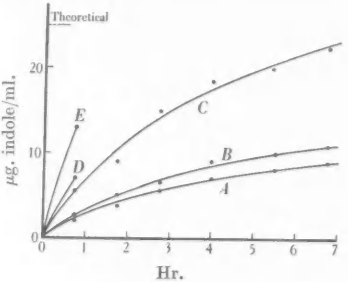

Fig. 3.

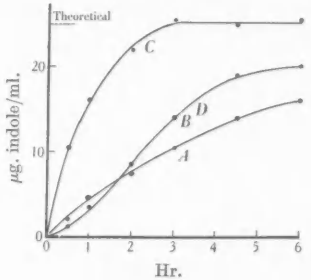

Fig. 4.

Fig. 3. Comparative activity of suspensions grown without tryptophan (*A*) and with tryptophan. *B* = tryptophan suspension diluted 1/25; *C* = 1/6; *D* = 1/4; *E* = undiluted.

Fig. 4. Effect of growth in glucose. *A* grown in lactate; *B* in glucose; *C* in lactate + tryptophan; *D* in glucose + tryptophan.

The effect of growth in glucose on the activity of the suspensions

It is a well-known phenomenon that the presence of glucose in a tryptophan-containing medium inhibits the production of indole by colon bacilli growing therein. Happold & Hoyle [1936] have concluded that this is due to a non-production of the "tryptophanase" enzyme in bacteria growing in the presence of glucose.

To test this possibility, flasks of the standard medium were prepared without, however, lactate; other ingredients were added as follows:

A, Medium + *M*/2 lactate 0·5 ml.
B, Medium + *M*/2 glucose 0·5 ml.
C, Medium + *M*/2 lactate 0·5 ml. + 0·175 % tryptophan 0·5 ml.
D, Medium + *M*/2 glucose 0·5 ml. + 0·175 % tryptophan 0·5 ml.

The cultures were grown for 20 hr. Those containing glucose were found to have given a smaller yield than those with lactate and thus the four suspensions

to be tested were made 1/3 the usual concentration. The centrifuged liquid of flasks *B* and *D* still contained glucose. Flask *C* contained quantities of indole equiv. 100% conversion of tryptophan. Flask *D* contained traces of indole, <1 μg./ml. and approximately 100% of unconverted tryptophan. The presence of glucose in the culture had therefore almost entirely inhibited the production of indole. Fig. 4 shows the action of the washed suspensions on tryptophan. It will be noticed that the suspension grown in glucose, after a preliminary delay which appears from other experiments to be constant, showed at least as much activity as that grown in lactate and that the presence of glucose had entirely inhibited the excess activity due to the tryptophan.

Thus it is possible to distinguish two "fractions" of activity in suspensions grown in the absence and presence of tryptophan.

(*a*) One fraction which is always present in *Bact. coli*, which does not depend for its production upon the presence of tryptophan in the external medium and which is unaffected by glucose therein. This fraction may be looked upon as "constitutive" in the sense of Karström [1938].

(*b*) Another fraction which is produced as a result of tryptophan in the external medium, which action is inhibited by the presence of glucose therein. This fraction may be called "adaptive".

It may readily be shown colorimetrically that the washed bacteria contain tryptophan which they have synthesized from NH_3 and thus it is possible that both fractions of activity result from an action of tryptophan.

The effect of indole upon the activity of suspensions

Three suspensions were made from cultures grown without added tryptophan. To each was added tryptophan to make a final conc. equivalent to 25 μg. indole/ml. To one was also added $\frac{1}{2}$ equiv. of indole and to another 1 equiv. Fig. 5 shows that the activity of the suspension was entirely inhibited by 1 equiv. indole and largely inhibited by $\frac{1}{2}$ equiv.

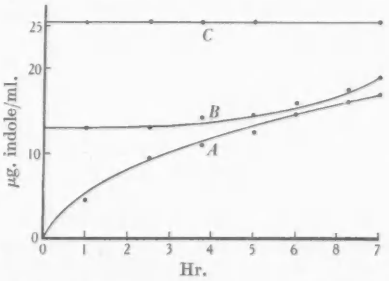

Fig. 5. Inhibition by indole. *A* = action of suspension on tryptophan; *B* on tryptophan + $\frac{1}{2}$ equivalent of indole; *C* on tryptophan + 1 equivalent of indole.

The same result was obtained when suspensions were used from cultures grown in tryptophan, but only when they were diluted to an activity similar to that of a suspension grown without tryptophan. When these suspensions were less diluted, more indole was required to produce a total inhibition. Thus the inhibitory action of indole depends upon the extent of enzyme surfaces present rather than upon the concentration of the substrate. The suspensions which had been proved to be inactive in the presence of tryptophan + indole

were shown to be potentially active by washing and resuspension in tryptophan. None of the indole derivatives already mentioned had any effect on the activity of suspensions under these conditions.

The effect of growth in the presence of indole and derivatives

In view of the effect of tryptophan in increasing the activity of suspensions of bacteria grown in a medium containing this substance, the actions of indole itself and of derivatives were tested.

As has been found by others the presence of indole in a culture is inhibitory to growth. In the present series when indole was added in equivalent concentration to the tryptophan, growth was delayed until the 2nd day and was not normally heavy until the 3rd. With indoleacrylic acid very little growth took place during the first 3 or 4 days but a heavy growth was found on the 4th or 5th. No other indole derivative was found to inhibit. By means of serial subculture in the inhibitory solutions, it was possible to banish the inhibition by indole and to reduce that by indoleacrylic acid so that full growth took place on the 2nd day. In the following experiment therefore the activities of suspensions were tested after 24 hr. growth except in the case of indoleacrylic acid, in which the bacteria were grown for 48 hr. The serial subcultures did not affect the general result of other similar experiments, but allowed a more exact comparison of activity to be made.

All cultures were prepared as before. Indole and its derivatives were dissolved in water or when necessary in $M/20$ NaOH in conc. equiv. 0·175% tryptophan and 0·5 ml. portions of these solutions were added to 10 ml. of medium, after sterilization by Seitz filtration.

The following derivatives were used: skatole, indolecarboxylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid, and indoleacrylic acid.

The suspensions were washed as usual and put up with tryptophan equiv. 25 μg. indole/ml.

Fig. 6 shows that an equal stimulation of the activity of the suspensions had resulted from growth in the presence of indole and indoleacrylic acid though the other compounds had no stimulatory effect.

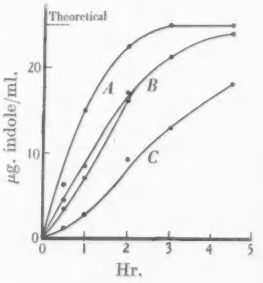

Fig. 6. Activity of suspensions grown in (*A*) tryptophan; (*B*) indole or indoleacrylic acid; (*C*) indole-pyruvic, -propionic, -carboxylic or -acetic acids, or in skatole, or with no addition.

The production of indole from indoleacrylic acid

It has been shown by Woods [1935, 2], Happold & Hoyle [1935] and others that suspensions of *Bact. coli* are unable to produce indole from indoleacrylic acid or any of the other derivatives mentioned. These results have been confirmed in the present work.

On the other hand in experiments in which the bacteria were grown in the presence of these substances, the result was different. Indole was formed from indoleacrylic acid added to the culture but not from the other derivatives even after 5 days' incubation.

The production of indole from indoleacrylic acid appears to have little relation to the amount of growth which has taken place. For instance in two experiments in which the growth was moderate in 24 hr. as a result of continuous subculture in indoleacrylic acid, the production of indole was only 1 μg./ml.

and <1 μg./ml. (theoretical 50 μg./ml.). On the 2nd day when growth was full, 2·5 and 4 μg./ml. had been produced. In another experiment using a strain unadapted to indoleacrylic acid the relation of growth to indole was as follows:

Days	Growth	Indole μg./ml.
1st	0	0·0
2nd	Trace	8·5
3rd	Trace	23·5
4th	+ + +	33·0

Under similar conditions the production of indole by suspensions grown in tryptophan would have been complete (50 μg./ml.) in less than 24 hr.

The mechanism by which the indole is produced is not clear but as a suspension of *Bact. coli* is unable to oxidize this substance directly, it would appear probable that under the conditions of growth *Bact. coli* is able slowly to synthesize tryptophan from indoleacrylic acid and that this is then oxidized to indole.

These results allow the conclusion that, whatever significance may be attached to the stimulation of activity by growth in tryptophan or indole, a similar significance need not be associated with the action of indoleacrylic acid, since this could be ascribed to the indole produced.

All workers are agreed that there is no experimental evidence to suggest that the oxidation of tryptophan to indole involves a passage through any of the theoretically possible intermediates tested. The present results with indoleacrylic acid are of interest in connexion with Raistrick's suggestion [quoted Cole, 1933] that this substance produced by a deamination of tryptophan may be the first step in the production of indole. If, as seems probable, *Bact. coli* can synthesize tryptophan from indoleacrylic acid the reverse process should also be possible, in agreement with Raistrick's idea. In practice it is not possible to demonstrate this action but this may merely be due to the much greater rapidity with which tryptophan is oxidized by another route. In any case the evidence still remains that indoleacrylic acid cannot be oxidized further and therefore this potential deamination of tryptophan can hardly be a step in the production of indole.

It appears to be generally held that the formation of indole from tryptophan probably takes place in steps catalysed by a series of enzymes. On the other hand if the detachment of the indole ring took place in one step under the influence of one enzyme it would be more easy to account for the phenomena noted here. The fact that growth in tryptophan or in indole leads to an increase in the enzyme activity is more in accordance with a view that these are substrate and end-product of one enzyme, rather than substrate and end-product of two different enzymes. Further the fact that indole completely inhibits the action on tryptophan is also reminiscent of the well-known inhibition of enzymes by their own end-products.

Summary

1. Suspensions of *Bact. coli* grown in the absence of tryptophan are always capable of oxidizing tryptophan to indole.

2. When grown in the presence of tryptophan, the activity is some twenty-five times greater.

3. The activity of the former suspensions is unaffected by the presence of glucose in the medium; the excess activity of the latter is entirely inhibited by glucose.

4. The activity of suspensions is inhibited by indole.

5. The activity of suspensions is increased by growth in the presence of indole or indoleacrylic acid. Other indole derivatives are inactive in this respect.

6. Indole is produced during growth in the presence of indoleacrylic acid, but not in the presence of other derivatives.

7. It is suggested that a growing culture can synthesize tryptophan from indoleacrylic acid.

I am much indebted to Mr D. D. Woods for helpful criticism and advice in the presentation of these results. I have also to thank Mr Woods and Dr F. C. Happold for the supply of some of the indole derivatives used.

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CCVIII. PHYSICAL PROPERTIES OF BUSHY STUNT VIRUS PROTEIN

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(*Received 23 July 1938*)

It has not hitherto been possible to determine accurately the molecular size or weight of any of the virus proteins. X-ray diffraction patterns show in the case of tobacco mosaic virus protein that the cross sectional area of the rod-shaped molecules is 20,100 sq. Å. [Bawden *et al.* 1936] and assuming the rod to be cylindrical this corresponds to a diameter of approximately 16 mμ. The same technique fails to give any indication of the length of the molecule and it is not at all certain that the molecular units have all the same length. Sedimentation, osmosis and diffusion measurements are also unsatisfactory when applied to the anisotropic plant viruses owing to the combination of large molecular size with gross asymmetry which they possess.

Some attempts have recently been made to apply to solutions of the anisotropic virus proteins equations relating the viscosity of a suspension of rods to the dimensions of the rods themselves. Frampton & Neurath [1938] and Lauffer [1938] deduced from viscosity measurements that the length of the tobacco mosaic molecule is 35–40 times the width. For a width of 16 mμ the length is therefore 560–640 mμ and the volume of the molecule can be calculated. If the density is 1·37 [Bawden & Pirie, 1937], the molecular weight by this procedure is $94–107 \times 10^6$, or approximately 100 millions. An alternative method of obtaining the molecular weight is to calculate the dissymmetry constant from the ratio of molecular length to width and to substitute this in the sedimentation equation. This method gives a weight of 42 millions [Lauffer, 1938]. Assuming, on the other hand, that the dissymmetry constant is 1·3, a value shared by a number of non-spherical proteins, a molecular weight of only 17 millions is obtained [Svedberg & Eriksson-Quensel, 1936]. The value of 1·3 is the lowest which could reasonably be proposed for a substance with the anisotropic properties of tobacco mosaic protein. Thus it seems that the molecular weight of the tobacco mosaic protein is more than 17 millions—probably considerably more—but less than 100 millions.

Recently a fully crystalline virus protein has been isolated [Bawden & Pirie, 1938, 1], the molecule of which appears to be spherical. This protein is obtained from the tomato plant infected with Bushy Stunt disease and is capable of transmitting the infection. It shows no anisotropy of flow and crystallizes in forms belonging to the cubic system. These properties suggest that the molecule is symmetrical and therefore adapted to exact measurements of size and weight by familiar physical methods. Our main object in the work described here has been to determine the size and weight of the virus protein molecule, but the opportunity has also been taken of investigating certain other properties, notably the electrophoretic behaviour.

The specimens of Bushy Stunt virus protein used in this work were prepared for us by Bawden & Pirie and a full description of the preparation is to

be found in their paper [1938, 2]. One specimen had been electrodialysed until the ash value, measured as sulphate, was 4 %. The solutions were colourless and water-clear by transmitted light.

Partial specific volume. This was determined pyknometrically at 25° and the values 0·743 and 0·734 obtained. The mean of 0·739 is approximately the same as Bawden & Pirie's value for tobacco mosaic virus protein and falls within the density range of the proteins as a class.

Sedimentation constant. Table I contains data from a series of sedimentation velocity determinations. These were carried out in the Svedberg equilibrium centrifuge at 21° and at a speed of 18,120 r.p.m. corresponding to a force of 20,000 times gravity in the middle of the fluid column. The ultraviolet light absorption technique was used for optical observation and the source of light was a high pressure mercury vapour lamp. A band in the ultraviolet was selected by means of chlorine and bromine gas filters. Exposure times of 15–20 sec. were used with Ilford Ordinary plates.

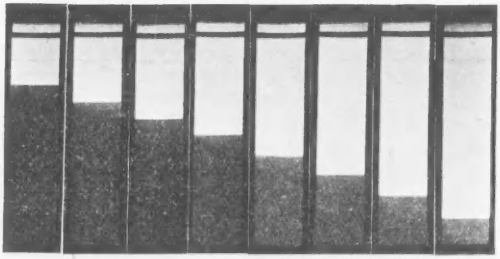

Fig. 1. A series of sedimentation pictures of the Bushy Stunt virus protein, 0·27 % in 0·02 *M* acetate buffer. Centrifugal force 20,000 g. 9 min. between each exposure.

Table I. *Sedimentation velocity of Bushy Stunt virus protein*

Protein concentration	Buffer composition	*p*H	$S_{W,\,20} \times 10^{13}$
0·11	0·01 *M* CH_3COONa; 0·01 *M* CH_3COOH	4·70	147
0·20	0·1 *M* citric acid; 0·018 *M* NaOH	2·40	138
0·20	0·0013 *M* KH_2PO_4; 0·0029 *M* Na_2HPO_4; 0·1 *M* NaCl	7·10	158
0·24	0·017 *M* CH_3COONa; 0·003 *M* CH_3COOH	5·46	153
0·25	0·003 *M* CH_3COONa; 0·017 *M* CH_3COOH	3·99	151
0·25	0·004 *M* CH_3COONa; 0·016 *M* CH_3COOH	4·12	142
0·25	0·05 *M* H_3BO_3; 0·014 *M* NaOH	8·70	141
0·26	0·006 *M* CH_3COONa; 0·014 *M* CH_3COOH	4·32	141
0·27	0·0005 *M* CH_3COONa; 0·0195 *M* CH_3COOH	3·40	143
0·29*	0·0013 *M* KH_2PO_4; 0·0029 *M* Na_2HPO_4; 0·1 *M* NaCl	7·10	145
0·34	0·01 *M* CH_3COONa; 0·01 *M* CH_3COOH	4·70	150

Mean $S_{W,\,20} = \mathbf{146} \times 10^{-13}$

* Electrodialysed prior to addition of buffer.

Fig. 1 shows a series of absorption pictures taken from one of the experiments in Table I, and typical of them all. The boundary is that of a perfectly homogeneous protein and shows a degree of sharpness which hitherto has only been obtained with some of the heavier haemocyanins [cf. Eriksson-Quensel & Svedberg, 1936, 1, 2]. One preparation which had been electrodialysed prior to the

addition of buffer showed the same perfect boundary sharpness as the others. The mean value of the sedimentation constant is 146×10^{-13}. The tobacco mosaic and cucumber virus proteins all give sedimentation constants higher than this, viz. $170–240 \times 10^{-13}$ [cf. Svedberg & Eriksson-Quensel, 1936; Wyckoff *et al.* 1937; Price & Wyckoff, 1938] but two plant viruses have smaller values, viz. latent potato mosaic (or "X" virus) for which $S_{W,20} = 113$ [Loring & Wyckoff, 1937] and tobacco ring spot virus for which $S_{W,20} = 115$ [Stanley & Wyckoff, 1937].

*p*H *stability.* From Table I it is seen that inside the range *p*H 2·4–8·7 the protein is homogeneous and unchanged in molecular size. This appears to be true for long periods at the hydrogen ion concentrations stated. After 1 hr. at *p*H 10 the protein consists of two components with sedimentation constants of 112 and 136×10^{-13}. Precisely the same splitting into components of 112 and 136 is found $\frac{1}{2}$ hr. after adjusting the *p*H to 1·3. After 48 hr. at this *p*H however only one boundary can be seen. This boundary is relatively sharp and sediments at a rate corresponding to $S_{W,20} = 66 \times 10^{-13}$.

The *p*H stability range is thus a comparatively wide one, viz. *p*H 2·40–8·70. It is of interest that nowhere inside this range are any signs to be found of a minimum solubility zone in which molecular aggregation occurs such as is shown by some of the anisotropic virus proteins [cf. Bawden & Pirie, 1937].

Sedimentation equilibrium. Three determinations have been made at different protein concentrations. The height of fluid column used was approximately 2 mm. and the equilibrium centrifuge was run at the lowest speed available, viz. 1500 r.p.m., corresponding to a force of 150 times gravity at the centre of the fluid column. The scale method based on Lamm's refraction studies [Lamm,

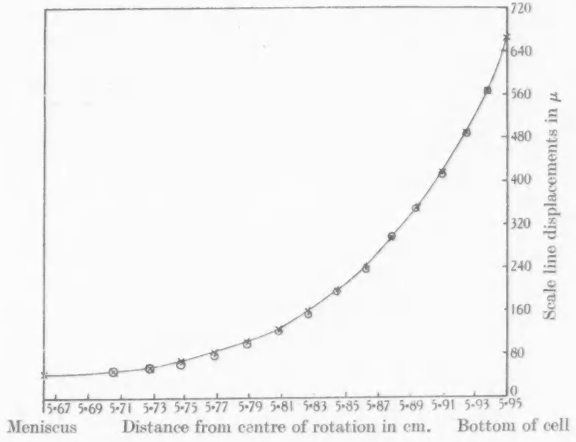

Fig. 2. ⊙ After 48 hours. × After 96 hours.

1937] was used, and the scale photographs were taken with light of wave-length 366 mμ. The superior accuracy of this method is especially apparent with the very steep equilibrium concentration gradients given by the heavier proteins. The temperature throughout was 20° and it was found that equilibrium was almost attained in 48 hr. and was complete after 96 hr. Fig. 2 shows the curves

obtained after 48 and 96 hr. in the same experiment. To obtain the concentration curve from this x, dn/dx curve a procedure recently described by Pedersen [1937] was used with advantage. The fluid column is divided into layers 0·02 mm. in thickness and the concentration in the middle layer is given a hypothetical value (C_0), for example the value of the protein concentration at the beginning of the experiment. By means of the concentration (or refraction) increments obtainable from the x, dn/dx curve the hypothetical mean concentration of an adjacent layer C_1, equal to $C_0 + dc/dx \times 0{\cdot}02$, is obtained and by repeating this procedure a series of mean concentrations corresponding to all the layers from the meniscus to the bottom of the cell is obtained. This enables a concentration curve to be constructed and the integral of this curve from the meniscus to the bottom of the cell should be equal to the total amount of protein in the cell. In order that this may be so a factor equal to the difference between the curve integral and the known amount of protein in the cell divided by the height of fluid column is then added (or subtracted) from the individual hypothetical layer concentrations, and a true concentration curve constructed. From the concentration values in each adjacent pair of fluid layers the molecular weight is calculated from the formula [Svedberg, 1926]

$$M_W = \frac{2RT \ln (C_2/C_1)}{(1 - V\rho)\,\omega^2 (x_2^2 - x_1^2)},$$

in which M_W is the molecular weight average and the other symbols have their customary significance.

The molecular weight may be determined by an alternative formula [cf. Pedersen, 1937], viz.

$$M_Z = \frac{2\,RT \log_n \left(\frac{Z_2 x_1}{Z_1 x_2}\right)}{(1 - V\rho)\,\omega^2 (x_2^2 - x_1^2)},$$

in which the Z values are scale line displacements in arbitrary units.

Table II. *Results of a typical sedimentation equilibrium experiment. Concentration of protein at start 0·335 % in 0·02* M. *Acetate buffer* pH *5·17. Cell 4 mm. thick. Speed 1530 r.p.m. Bottom of cell 5·95 cm. from axis of rotation* $t = 20°$

				Molecular weight × 10^6	
x	z in μ	dc/dx	C %	Weight-average (M_W)	Z-average (M_Z)
5·95	662	11·1	1·054		
5·93	515	8·64	0·962	7·22	7·55
5·91	412	6·91	0·807		
5·89	329	5·52	0·682	7·36	6·80
5·87	264	4·43	0·583		
5·85	211	3·55	0·503	7·70	6·72
5·83	168	2·83	0·440		
5·81	133	2·23	0·389		
				8·26	7·27
				Mean **7·64**	**7·09**

Table II shows the results of a typical experiment and it will be seen that the variation in M_W or M_Z values with height in the cell is comparatively small. This is a reliable criterion that equilibrium has been established and that we are concerned with a homogeneous protein.

In Table III are shown the mean M_W and M_Z values for three equilibrations. The mean molecular weight value of all the determinations is 7,600,000.

Table III. *Results of three sedimentation equilibrium determinations on Bushy Stunt virus protein*

Protein concentration	Buffer composition	*p*H	Mean molecular wt. × 10^6	
			M_W	M_Z
0·335	0·015 *M* CH_3COONa; 0·005 *M* CH_3COOH	5·17	7·64	7·09
0·220	0·015 *M* CH_3COONa; 0·005 *M* CH_3COOH	5·17	8·15	7·22
0·270	0·001 *M* CH_3COONa; 0·039 *M* CH_3COOH	3·40	8·01	7·42
		Mean	7·93	7·24

Size and weight of the molecule. The sedimentation constant is defined as the molecular velocity under unit force in water at 20°. Since the force of gravity on a spherical particle is $\frac{4}{3}\pi r^3(\rho_v - \rho_s)$, the force required to produce unit velocity is

$$\frac{4\pi r^3(\rho_v - \rho_s)}{3 \times S_{W,20}},$$

where ρ_v is the density of the protein and ρ_s is the density of the solvent.

According to Stokes' law this force is equal to $6\pi\eta r$, where η is the viscosity of the medium.

$$6\pi\eta r = \frac{4\pi r^3(\rho_v - \rho_s)}{3\,S_{W,20}},$$

$$r^2 = \frac{9\eta\, S_{W,20}}{2(\rho_v - \rho_s)}.$$

Substituting $S_{W,20} = 146 \times 10^{-13}$ and $\rho_v = 1{\cdot}353$ for Bushy Stunt protein we find the radius of the molecule to be 13·7 mμ and the weight 8,800,000.

The agreement between the molecular weight obtained from sedimentation equilibrium measurements and from sedimentation velocity measurements assuming Stokes' law indicates that the molecule does not depart markedly if at all from a symmetrical shape.

Electrophoretic mobility. Measurements of the electrophoretic mobility of the virus protein were made with the Tiselius apparatus [Tiselius, 1937], the migration of the protein boundary being followed photographically by the "Schlieren" method (Fig. 3). A sodium vapour lamp was used as a light source and exposures made on Ilford Panchromatic plates.

The mobility was measured at 0° in acetate buffers, total acetate 0·02 *M*, the protein concentration being maintained between the limits 0·25–0·35 g./100 ml. Corrections were applied for the viscosity effect of the buffer salts, and for the hydrostatic displacement [Tiselius, 1930]. Both of these corrections amounted to less than 1 %.

The material showed the behaviour characteristic of a homogeneous substance, a single sharp Schlieren band being maintained throughout the experiments over the range of *p*H studied.

Details of the mobility experiments are given in Table IV and Fig. 4.

Table IV. *Electrophoretic mobility of Bushy Stunt virus protein*

*p*H	Mobility (u) cm.$^{-2}$ V.$^{-1}$ sec.$^{-1}$ × 10^{-5}	Direction
3·80	1·79	Cathodic
3·99	0·73	,,
4·12	0·15	Anodic
4·32	0·58	,,
4·70	1·73	,,
5·17	3·46	,,
5·47	4·59	,,

The isoelectric point of the virus protein was found to be $p\mathrm{H}_0 = 4{\cdot}11$ and the slope of the mobility curve at the isoelectric point, $du/d\,(p\mathrm{H}_0) = 5{\cdot}0 \times 10^{-5}$.

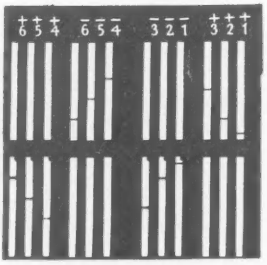

Fig. 3.

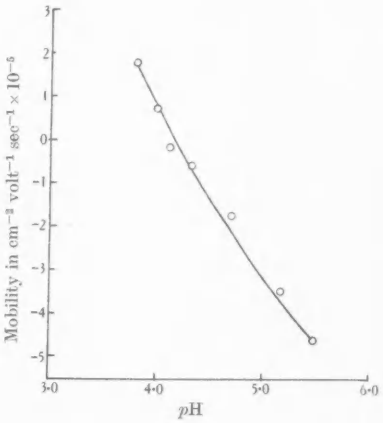

Fig. 4.

Fig. 3. Electrophoretic migration of Bushy Stunt virus protein in 0·02 *M*. Acetate *p*H 6·2. Potential gradient 9·5 V./cm. A single sharp boundary is present in all the exposures.

Fig. 4. Mobility-*p*H curve for Bushy Stunt virus protein in 0·02 *M* acetate buffer.

Specific refractive increment and optical dispersion. The refractive increment of a solution of the protein in 0·01 *M* NaCl at the isoelectric point was measured for three wave-lengths in the mercury spectrum using a photographic technique [cf. *Handbuch der Physik*, **18**, 668, 1927]. The protein concentration was determined by drying to constant weight *in vacuo* at room temperature. The results are given in Table V.

Table V. *Specific refraction increment of Bushy Stunt virus protein at different wave-lengths. $T = 23°$.*

Wave-length mμ	Specific refraction increment
366	0·00178
436	0·00170
546	0·00164

Summary

The partial specific volume, sedimentation constant, molecular weight, electrophoretic mobility, isoelectric point and specific refraction increment of the crystalline nucleoprotein causing Bushy Stunt disease in the tomato plant have been determined. The protein is exceptionally homogeneous both with respect to size and charge and no evidence of an asymmetrical molecular shape could be found.

One of us (R. A. K.) is in receipt of a grant from the Medical Research Council.

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CCIX. THE MANOMETRIC DETERMINATION OF AMINO-ACIDS

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In a recent number of this *Journal* Mason [1938] has published under the above title a method depending upon the measurement, in the Van Slyke-Neill [1924] apparatus, of the CO_2 evolved from the carboxyl groups of amino-acids boiled with ninhydrin. This method had already been published by Van Slyke & Dillon [1936], in a paper which Mason quotes only as having "suggested" the method "as feasible". Although Van Slyke & Dillon's paper was presented as a preliminary report, the method was described in sufficient detail for exact repetition, and the nature of the results with different types of amino-acids, with polypeptides, and with other types of organic acids was stated. A supplementary description, with some improvements, was later published by Van Slyke & Dillon [1938] in Sørensen's Jubilee Volume, 3 months before the appearance of Mason's report. The method as republished by Mason [1938] differs in no significant detail from that originally outlined by Van Slyke & Dillon [1936].

Because it depends on the presence of both COOH and NH_2 or C—NH—C groups in neighbouring positions, the method appears to be the most specific yet devised for measurement of amino-acids in biological mixtures. For example, D. A. MacFadyen finds in the writer's laboratory that it can be applied directly to serum without removal of proteins. The full report on the method, including the results of Dillon and MacFadyen on which it and its applications are based, will appear in the near future.

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CCX. PHENAZINE COMPOUNDS AS CARRIERS IN THE HEXOSEMONOPHOSPHATE SYSTEM

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Certain phenazine compounds resemble the flavins in that they can readily form semiquinones, to which property, generally very rare in dyestuffs, Michaelis *et al.* [1936, 1, 2] attach much importance in determining the catalytic activities of the flavin enzyme and vitamin B_2.

Phenazine methiodide [Dickens, 1936, 1] like pyocyanine [Friedheim, 1934] and certain flavin compounds [Laser, 1934] has an action on tissue metabolism which is quite different from that of most of the usual oxidation-reduction dyestuffs [Dickens, 1936, 1]. In tissues exhibiting strong aerobic glycolysis, particularly tumour tissue, the respiration is increased and the aerobic glycolysis falls in the presence of these compounds which thus appear to exert a control of the Pasteur reaction [cf. Dickens, 1936, 2]. While the action of the simpler phenazines is rapid, enabling its detection in short experiments by the tissue-slice method, that of the flavins can only be demonstrated in tissue culture experiments, presumably owing to slow absorption or slow transformation into the active carrier. On the other hand the easily diffusible phenazines cause some inhibition of the anaerobic glycolysis also [Dickens, 1936, 1], so that this is not a simple augmentation of the Pasteur effect. This group of substances produces such unusual effects that an attempt has been made to elucidate the mechanism of their action by a study of the catalytic role of the phenazines in an isolated enzyme system. Weil-Malherbe [1937, 2] has studied the action of phenazine methochloride and pyocyanine in the α-hydroxyglutaric system.

In the experiments described below we have prepared some new phenazine compounds and used them as substitutes for the flavin enzyme of Warburg in the hexosemonophosphate system, comparing their catalytic activities with those of other carriers. We have also measured the oxidation-reduction potentials of a number of phenazines for correlating our results. The experiments reveal new aspects of the common features of the phenazines and flavin compounds, and it is shown that in the hexosemonophosphate system the catalytic activity of some of these compounds much surpasses that of the ordinary oxidation-reduction dyestuffs studied.

Green *et al.* [1934] used pyocyanine as carrier in this system in their studies on carrier-linked reactions. Later Ogston & Green [1935] stated their objections to the description of the flavin-phosphate-protein complex as an enzyme. In the following we have retained the term flavin enzyme, since the question cannot be regarded as settled.

Enzyme and substrate preparations

Enzyme preparations. Many of the experiments were made on preparations of "Zwischenferment" [Negelein & Gerischer, 1936] and coenzyme II of purity 1, generously supplied by Prof. Warburg, who also gave us a sample of flavin

enzyme purified by the $CHCl_3$ method [Warburg & Christian, 1933] containing in the 10% solution $4{\cdot}1 \times 10^{-8}$ g.mol. pigment per ml. as estimated by Prof. Warburg. For use, 0·1 mg. dry Zwischenferment per vessel was dissolved in 2 ml. *M*/100 phosphate buffer. For other experiments we used a simple enzyme preparation made from Lebedew fluid prepared from Löwenbräu, Munich, bottom beer yeast as follows. The finely ground yeast, air-dried, was incubated for $2\frac{1}{2}$ hr. with 3 vol. water at 35°. After centrifuging, the clear fluid was diluted with 5 vol. distilled water and 0·15 *N* acetic acid was added with stirring and ice cooling, to give *p*H 4·6 (the volume of acetic acid required is about half the vol. of Lebedew fluid taken). The white precipitate was collected at the centrifuge, washed with 0·02 *M* acetate buffer *p*H 4·6, and dried quickly *in vacuo* over P_2O_5. The yield was about 2·5 g. per 250 g. dried yeast. If necessary, the enzyme may be dissolved in 0·02 *N* ice cold NaOH and, after centrifuging from some insoluble residue, reprecipitated by acetic acid as before. It may be kept in the refrigerator for several weeks in the dry state without loss of activity. For use it was dissolved (10–20 mg. per vessel) in ice cold *N*/100 NaOH or *M*/100 phosphate buffer with addition of NaOH to give a final solution of the required *p*H, and centrifuged. Although this enzyme is much cruder than the Warburg material, with the quantity taken the blank was zero or very small and the rates of O_2 uptake were equal with the two preparations.

Coenzyme II of purity 0·2 was prepared from horse blood corpuscles by the method of Warburg & Christian [1936], "1 and 2 Schritte". Of this 0·1 mg. per vessel used instead of 0·02 mg. of the preparation of purity 1 gave the same rate of O_2 uptake.

Substrate. Ba hexosemonophosphate was prepared from the action of dialysed muscle extract on glycogen [Ostern *et al.* 1936]. It was found essential to allow full digestion to reduce the glycogen content to a minimum, but traces nevertheless remained. Prof. Robison very kindly analysed a specimen as follows. Found: total P 7·4%; inorg. P 0·1%; H. and J. reduction 32·9; I.V. 32·3; Seliwanoff 5·6; $[\alpha]_{5461}$ 23·4°; glycogen +. Calc. for $C_6H_{11}O_9PBa$: P 7·85%. The analysis is that of a mixed hexosemonophosphate [cf. Robison, 1932] containing a little glycogen.

For the preparation of the solution of the Na salt, 0·145 g. Ba salt was ground with 0·354 g. $Na_2SO_4, 10H_2O$ and diluted to 5 or 10 ml. after the addition of 0·125 ml. *N* HCl. The centrifuged solutions contained respectively 0·18 or 0·09 *M* Na hexosemonophosphate (P analysis).

Phenazine compounds

Methylphenazonium salts. Phenazine methosulphate was prepared by the addition of an equivalent of dimethyl sulphate, freshly distilled *in vacuo*, to phenazine in nitrobenzene solution [Kehrmann, 1913; Hillemann, 1938] and crystallized quickly from alcohol, M.P. 167°. Methylphenazonium chloride was obtained as yellow needles by repeated crystallization of the methosulphate in presence of KCl (found: Cl 15·1; calc. for $C_{13}H_{11}N_2Cl$ 15·4%). This salt has previously been described as green by Browning *et al.* [1922] who prepared it by dissolving in HCl the product obtained by action of alkalis on phenazine methosulphate. This must be regarded as impure, for methylphenazonium salts decompose in alkaline solutions [McIlwain, 1937, 2; Hillemann, 1938].

Methylphenazonium phosphate was prepared by the addition of excess Na_2HPO_4 to the methosulphate, each in the minimum amount of water; the salt separated as brown prisms.

Semiquinoid methylphenazonium phosphate was obtained in fine green needles by warming the methosulphate in alcohol with excess phosphoric acid: I_2 equiv. [McIlwain, 1937, 2] 299; calc. for $C_{13}H_{11}N_2PO_4$ 292. Partial reduction of the methylphenazonium salts under warm acid conditions has been previously recorded [Kehrmann, 1914].

2-Aminophenazine methosulphate was prepared according to Kehrmann [1913] and the remainder of the compounds by the methods previously described [McIlwain, 1937, 1, 2].

Potentiometric titrations

The potentials were determined by titration of *M*/2000–4000 solutions in phosphate buffer with *c.* *M*/200 chromous acetate, prepared by diluting the satd. aqueous soln. with 2 vol. de-aerated water in a storage burette of the type described by Cohen & Phillips [1929]. The electrode vessel was kept at $30 \pm 0{\cdot}1°$ and de-aerated and stirred with N_2 freed from O_2 by hot Cu. Potentials were read from 3 gold-plated electrodes which normally agreed within 1 mv. Quinhydrone in 0·1 *N* HCl was used as the standard half-cell.

The titration curves showed no evidence of 2-stage oxidation-reduction processes over the *p*H range investigated, but slight intermediate colours were in some cases observed. The initially yellow solutions of the alkyl phenazonium salts become slightly yellow-green before separation of the colourless dihydro-compound on complete reduction. The reduced keto compound was pale cream coloured and insoluble; the reduced sulphonates and aminophenazine were soluble and almost colourless.

$Na_2S_2O_4$ was initially used as the reducing agent but though this gave normal titration curves, the results were not considered reliable as the titration could not be repeated after oxidation of the reduced solution. Progressively more positive values were obtained on repetition. A secondary reaction had evidently occurred as the solution showed a green fluorescence and the reduced compounds were no longer insoluble. In the case of phenazine methosulphate the values approached those of the sulphonates. It is understandable that such sulphonates should be produced from the sulphite formed during reduction, as their preparation from phenazonium salts and sulphite has been described [McIlwain, 1937, 2]. The initial values in titration with $Na_2S_2O_4$ were however substantially correct; Preisler & Hempelmann [1937] have used $Na_2S_2O_4$ in such titrations.

The compounds investigated considerably increase the range of oxidation-reduction potentials observed in the phenazine series (Table I). The simple

Table I

Compound	E_h, *p*H 7 30°	Authority
Na *N*-methylphenazoniumdisulphonate betaine	+0·230	Present investigations
N-Methylphenazonium sulphonic acid betaine	+0·130	,,
Phenazine methosulphate	+0·080	,,
Phenazine ethosulphate	+0·055	,,
4-Keto-*N*-methylphenazine (pyocyanine)	−0·034	[Friedheim & Michaelis, 1931]
4-Keto-*N*-ethylphenazine	−0·055	Present investigations
Phenazine-1-carboxylic acid amide (chlororaphin)	−0·115	[Elema, 1933]
2-Amino-*N*-methylphenazine methosulphate	−0·145	Present investigations
2-Keto-*N*-methylphenazine	−0·165	[Preisler & Hempelmann, 1937]
1-Hydroxyphenazine	−0·173	[Michaelis, 1931]
2-Hydroxyphenazine	−0·214	[Preisler & Hempelmann, 1937]
2:7-Diamino-*N*-phenylphenazonium chloride (phenosafranine)	−0·252	[Stiehler *et al.* 1933]
2-Amino-7-dimethylamino-2-methylphenazine (neutral red)	−0·325	[Clark & Perkins, 1932]

phenazine quaternary salts and sulphonic acids are much more positive than previously studied compounds, some examples of which are quoted. Table I also affords an excellent example of correlation of E_h with the electronic properties of the substituent groups, electron-attracting groups producing more positive and electron-repelling groups more negative potentials.

Phenazines as carriers

Methods. The O_2 uptake was measured in Warburg manometers at 37·5°. Each vessel contained in the main part 2 ml. enzyme solution, 0·1 mg. coenzyme of purity 0·2 (in some experiments 0·02 mg. of purity 1), 0·3 ml. $M/10$ hexose-monophosphate. The side bulb held the solution of carrier in 0·2 ml. The inner cup contained 0·2 ml. N NaOH and the gas space was filled with pure O_2. Usually these conditions were adhered to, but sometimes slight variations were made. The O_2 uptake during the first hr. was measured; it continued regularly for a longer period provided that the substrate concentration did not fall too much. Readings were corrected for a blank without substrate. In the absence of added substrate the blank was small, a few μl. per hr., except when large quantities of autoxidizable carrier were present. This autoxidation is much reduced by working at a slightly acid reaction, pH *c.* 6·5, without affecting the catalytic activity, and this was done in later experiments; other experiments were at pH 7–7·5. The rates of O_2 uptakes were the same with the various combinations of enzyme and coenzyme used, within fairly close limits, since both these components were present in excess and the amount of carrier was ordinarily the limiting factor. The carriers were dissolved in water and where necessary neutralized. A few (Table II, Nos. 12, 19) were sparingly soluble and a suspension containing the wt. shown was placed in the side bulb. Flavin enzyme was dissolved in 10 vol. of water and centrifuged to remove a trace of denatured protein. Amounts of carrier are expressed in mg. and μl. (1 mM = 22400 μl.). In the case of flavin enzyme 1 mM prosthetic group, photometrically determined, is taken as 22400 μl.

Results. These are shown in Table II.

The results may be grouped according to the rate of O_2 uptake.

Group 1. Maximum activity (*c.* 200 μl./hr.) is given by only a few substances. These were: phenazine methosulphate, methochloride and ethosulphate (amount of carrier *c.* 20 μl.) and flavin enzyme (Nos. 3, 4, 5 & 10).

Group 2. Of less but still marked activity were the mono- and di-sulphonic acid derivatives of N-methylphenazine (Nos. 1 and 2), pyocyanine (No. 8) and its ethyl homologue (No. 9) whose activity exceeds that of pyocyanine, the 2-amino derivative of N-methylphenazine methosulphate (No. 14) and the red oxidation product of N-methylphenazine quaternary salts (No. 15) and its ethyl homologue (No. 16). In amounts of from 22–50 μl. this group caused O_2 uptakes of from 40–80 μl./hr. It is noteworthy that all the compounds of Groups 1 and 2, with the exception of the flavin enzyme, are derivatives of N-alkylphenazines.

Group 3. Among non-phenazine compounds investigated none approached in activity the simple phenazine quaternary salts. Brilliant cresyl blue and methylene blue showed most activity in this group, being comparable with the lower members of Group 2 (50 μl. dye caused *c.* 30–45 μl. O_2/hr.).

Group 4. The remaining compounds studied were of low or zero activity (with chlororaphin and dimethylalloxazine the lack of activity might have been due to their insolubility). Methyl Capri blue, though in the same region of potential as active carriers, belongs to this group. Acridine methochloride (No. 22, Table II) was quite inactive as carrier, though chemically it differs from phenazine methochloride only in the substitution of a CH group for a N atom.

Table II

No.	E_h, pH 7	Substance	Amount per vessel mg.	Amount per vessel µl.	O_2 uptake* µl. (1st hr.)	Turnover no. (Average over 1st hr.) Mol. O_2/min. / Mol. catalyst × 0·5
1	+0·230	Sodium *N*-methylphenazonium disulphonate betaine	0·5	30	79	0·088
2	+0·130	*N*-Methylphenazonium sulphonic acid betaine	0·5	37	68	0·061
3	+0·080	Phenazine methosulphate	0·3	22	204	0·310
4	—	Phenazine methochloride†	0·3	27	206	0·260
5	+0·055	Phenazine ethosulphate	0·3	19	190	0·330
6	+0·045	Brilliant cresyl blue	1·5	106	71·5	0·022
			0·75	53	47	0·029
			0·5	35	36	0·034
7	+0·011	Methylene blue	1·5	90	34	0·013
			1·0	60	28	0·016
			0·75	45	27	0·021
			0·5	30	27	0·030
			0·25	15	26	0·057
8	−0·011	Pyocyanine hydrochloride† (4-keto-*N*-methylphenazine)	0·45	45	47	0·030
9	−0·055	4-Keto-*N*-ethylphenazine	0·3	30	80	0·090
10	−0·060	Flavin enzyme	(20)‡	0·18	215	39·600
			(5)‡	0·045	110	81·400
11	−0·060	Methyl Capri blue	0·3	22	13	0·020
12	−0·115	Chlororaphin (satd.)	<0·3	<30	15	<0·020
13	−0·142	Gallophenin	0·5	37	4	0·003
14	−0·145	2-Amino-*N*-methylphenazine methosulphate	0·3	21	40	0·063
15	−0·165	2-Keto-*N*-methylphenazine	0·5	50	69	0·047
			0·14	14	35	0·083
16	−0·180	2-Keto-*N*-ethylphenazine	0·3	28	45	0·053
17	−0·180	Lactoflavin	0·3	20	0·5	0·001
18	−0·252	2:7-Diamino-*N*-phenylphenazonium chloride (phenosafranine)	0·3	21	0	0·000
Other compounds:						
19	—	6:7-Dimethyl alloxazine (satd.)	<0·1	< 7	0	0·000
20	—	Nicotinamide methiodide	0·5	42	1	0·000
21	—	Phenazine-1-carboxylic acid amide	0·5	49	4	0·003
22	—	Acridine methochloride	0·3	29	0	0·000
23	—	1:2:3:4-Tetrahydrophenazine methosulphate	0·3	20	25	0·040
24	—	Aneurin	2·0	133	0	0·000
25	—	Thiochrome	0·6	51	0	0·000

* Corrected for blank, if any. † See Fig. 1.
‡ Wt. of crude substance taken; flavin content photometrically determined.

The catalytic effect is dependent on the concentration of carrier. This is clearly shown by the data of Table II for brilliant cresyl blue and methylene blue. The turnover no. in the last column shows the decreasing activity with increasing concentration of these dyes. Since this is not here due to the limiting capacity of the enzyme system, both being feeble catalysts for this system, it is apparently due to a poisoning effect of these dyes when present in high concentration.

A different effect is seen with the *N*-alkylphenazines. Here, owing to their greater activity, the O_2 uptake is limited by the enzyme system when the amount of these catalysts is much increased. Fig. 1 shows the activity-concentration curves of phenazine methochloride and pyocyanine. (The preparation of enzyme was actually made to study the oxidation of phosphohexonate, the curves for

which are also included for comparison in Fig. 1. The rather high concentration, $M/30$, of phosphate buffer has inhibited the hexosemonophosphate oxidation more than that of phosphohexonate [see Dickens, 1938].)

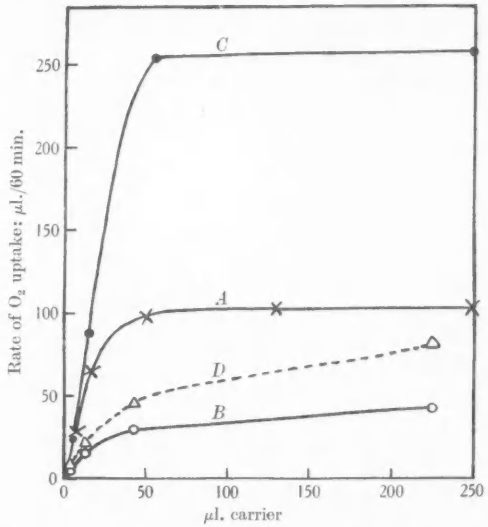

Fig. 1. Comparison of phenazine methochloride and pyocyanine as carriers. Curve A: phenazine methochloride, hexosemonophosphate. Curve B: pyocyanine, hexosemonophosphate. Curve C: phenazine methochloride, phosphohexonate. Curve D: pyocyanine, phosphohexonate. 20 mg. acetate-precipitated enzyme, 2 ml. $M/30$ phosphate buffer, 0·1 mg. coenzyme (purity 0·2), 0·3 ml. substrate.

Activity relative to flavin enzyme

While the absolute values of O_2 uptake with the hexosemonophosphate system when phenazines are added attain those given by flavin enzyme as carrier, the turnover numbers (T)[1] in Table II show clearly that their efficiency is very much less. For the flavin enzyme, $T = 40$–80 mol. O_2/0·5 mol. prosthetic group min. with our enzyme preparation. Warburg & Christian [1933] give the values as 25–50 mol. O_2/mol. prosthetic group min. In contrast with this high activity, the non-colloidal carriers listed in Table II give much lower T-values, the most active phenazonium salts having only about 1/250th of that of the flavin enzyme, while pyocyanine in similar concentration is about ten times less active still.

E_h *relationships: "specificity"*

The E_h of the simple N-alkylphenazonium salts varies from 0·08 to 0·06. That of the flavin enzyme is recorded by Kuhn & Boulanger [1936] as −0·06 V. (i.e. 0·12 V. more positive than lactoflavin). Although intermediate between

[1] For the calculation of the turnover no. it is important to know if the O_2 taken up is partly lost in side reactions (e.g. H_2O_2 formation or coupled oxidation) or if it is wholly reduced by the H transported by the catalyst concerned. Only in the latter case is the turnover no. given by the ratio: mol. O_2/0·5 mol. catalyst min. With 100% yield of H_2O_2 the equation becomes T = mol. O_2/mol. catalyst min., as used by Warburg & Christian [1933].

these in potential methylene blue and brilliant cresyl blue are inferior catalysts for this reaction. Hence thermodynamic considerations alone do not govern the efficiency of the carriers tested in this system. It is also noteworthy that other phenazines (Nos. 1, 2, 14, 16, Table II) whose potentials lie outside those of the alkylphenazonium salts and flavin enzyme in both positive and negative directions, are somewhat more efficient catalysts than the dyes methylene blue and cresyl blue, although according to their potentials these dyes should have activities between that of the most active alkylphenazines and flavin enzyme. There is thus a certain limited "specificity" in the chemical nature of the active carriers for this system. It may be possible to explain this structural specificity in terms of semiquinone formation [Michaelis *et al.* 1936, 1, 2]. The activity may be determined by the concentration of semiquinone in the half-reduced form at the reaction of the system.

Catalytic activities of phenazines added as semiquinones

Semiquinonoid methylphenazonium phosphate and 1:2:3:4-tetrahydrophenazine methosulphate [McIlwain, 1937, 1] were tested. The activity of the semiquinonoid and ordinary forms of the compounds was the same, but as the bright green solution of semiquinones rapidly changed to yellow on addition to the enzyme system, it is evident that only a trace of semiquinonoid compound could have been present, and this of course is no evidence against the importance of semiquinone formation.

Qualitative differences in effect of phenazines and flavin enzyme

Whereas the enzyme system used attacked Neuberg & Robison esters at about the same rate in presence of flavin enzyme as carrier, the Neuberg ester was much

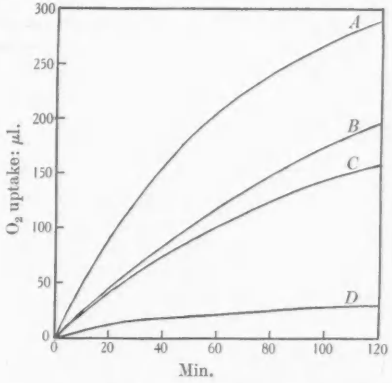

Fig. 2. Curve A: Robison ester + 0·3 mg. phenazine methochloride, 0·3 mg. phenazine methosulphate, or 10 mg. (crude) flavin enzyme. Curve B: Neuberg ester + 5 mg. flavin enzyme. Curve C: Robison ester + 5 mg. flavin enzyme. Curve D: Neuberg ester + 0·3 mg. phenazine methochloride. 0·1 mg. Zwischenferment in 2·3 ml. *M*/100 phosphate buffer, + 0·02 mg. coenzyme II of purity 1, + 0·4 ml. *M*/6 phosphoric ester.

less rapidly attacked than the Robison ester, when phenazine methochloride was used instead of flavin enzyme (Fig. 2).

Neuberg ester was prepared from Ca hexosediphosphate (B.D.H.) by hydrolysis with oxalic acid [Neuberg, 1918]. The Ca salt thus obtained was decomposed by the calc. amount of oxalic acid and after removal of Ca oxalate neutralized with NaOH.

As yet the mechanism of the oxidation of Neuberg ester has not been examined. It is therefore difficult to explain this difference, which may be accounted for either by the presence of some other enzyme than the flavin one in the crude preparation of flavin enzyme, or less probably by some inhibitory action of the phenazine compound on a component of the fructosemonophosphate-oxidizing system. That phenazine compounds can exert an inhibitory action on enzymes is readily shown. Weil-Malherbe [1937, 1] showed that succinic dehydrogenase is inhibited by pyocyanine and phenosafranine. The action of phenazine methochloride on carboxylase and succinic dehydrogenase is shown in Table III.

Table III. *Inhibition of carboxylase and succinic dehydrogenase*

Carboxylase. 50 mg. yeast carboxylase [Axmacher & Bergstermann, 1934] in 0·9 ml. water per vessel, with 0·3 ml. phosphate buffer *p*H 6·5 and 1 ml. addition of dye (or water). Side bulb contained 0·2 ml. *M*/10 Na pyruvate. Gas space, N_2. Temp. 25°.

Addition:	Water	2 mg. pyocyanine	2 mg. phenazine methochloride	2·6 mg. phenosafranine	2·5 mg. cresyl blue	1·7 mg. 2-keto-*N*-methylphenazine
			CO_2 evolved (μl.)			
30 min.	195	192	95	154	182	192
60 ,,	228	223	112·5	198·5	216·5	235
120 ,,	250	242	121·5	232	242	259

Inhibition by *M*/300 phenazine methochloride = 50%; others, none.

Succinic dehydrogenase. 50 mg. muscle enzyme, suspended in 1·5 ml. *M*/10 succinate in *M*/50 phosphate buffer *p*H 7·4; 0·5 ml. cytochrome. Side bulb contained dyes 0·4 ml. *M*/50. Air, 37·5°; 0·2 *N* NaOH in inner cup. Phenazine methochloride readings corrected for autoxidation of carrier.

Addition	Water	Phenazine methochloride	Pyocyanine	Phenosafranine
O_2 uptake μl. (60–120 min.)	98	76	32	28
Inhibition %	—	23	68	70

In these two cases very different actions are seen; phenazine methochloride in *M*/300 concentration inhibits carboxylase by 50% but has little action on succinic dehydrogenase. On the other hand pyocyanine and phenosafranine, which strongly inhibit succinic dehydrogenase [Weil-Malherbe, 1937, 1] have little effect on carboxylase. Michaelis & Smythe [1936] have already drawn attention to the poisoning by a similar concentration of pyocyanine of glucose fermentation in Lebedew fluid and its restoration by addition of a carboxylase preparation.

Action of iodide ion

In the earlier experiments with this system we used phenazine methiodide as in the earlier tissue experiments [Dickens, 1936, 1]. It was found with the hexosemonophosphate system that, using the methiodide as carrier, the reaction came to a standstill after 20–40 min. This was due to the I^- of the quaternary salt, since it did not occur with phenazine methochloride and the action of phenazine methochloride and flavin enzyme is inhibited by small quantities of NaI (Fig. 3).

The inhibition is perhaps due to an interaction either of I^- or of I_2 with the coenzyme, for it appeared to be partially removed by addition of fresh coenzyme to the system. The inhibition by I^- does not appear to have been observed before.

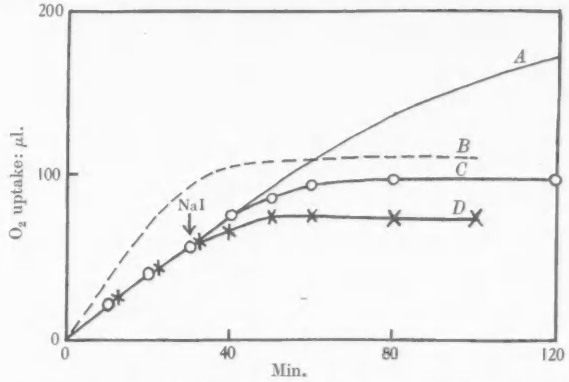

Fig. 3. Hexosemonophosphate oxidation. Curve A: 0·15 mg. methylphenazonium salt* or 5 mg. flavin. Curve B: 0·16 mg. phenazine methiodide. Curve C: 5 mg. flavin enzyme +0·1 mg. NaI, $2H_2O$ tipped in at 30 min. Curve D: 0·15 mg. methylphenazonium salt* +0·3 mg. NaI, $2H_2O$ tipped in at 30 min.

Action of phenazine methochloride on tissue metabolism

In view of the above results the action of the methochloride on tissue slices was tested. The results do not differ greatly from those seen with the methiodide [Dickens, 1936, 1], but as the fall of anaerobic glycolysis is less with the methochloride, the apparent partial restoration of the Pasteur mechanism is better seen (Table IV). The results with the red oxidation product of *N*-methylphenazines (2-keto-*N*-methylphenazine) were obtained with a pure specimen made synthetically [Kehrmann, 1924], and not by autoxidation, since free phenazine always results by the latter method; the specimen used was freely soluble in

Table IV. *Action of phenazine methochloride and 2-keto-*N*-methylphenazine on metabolism of Jensen sarcoma slices*

Warburg two-vessel technique, $NaHCO_3$-glucose Ringer. Temp. 37·5°. $CrCl_2$ in anaerobic vessels.

	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
		Control			Phenazine methochloride (10^{-3} *M*)		
Exp. 1	0–60	−10·8	+22·2	+40·8	−13·5	+ 8·6	+45·6 (yellow)
	60–120	—	—	+36·4	—	—	+31·9 (colourless)
	120–180	—	—	+35·0	—	—	+25·0 (colourless)
Exp. 2	0–30	−14·3	+21·4	—	−28·0	+12·1	—
	30–60	−10·1	+18·6	—	−10·3	+ 4·1	—
	60–90	− 9·1	+18·0	—	− 9·6	+ 3·1	—
		Control			2-Keto-*N*-methylphenazine (10^{-3} *M*)		
Exp. 3	0–60	−16·8	+20·4	+28·2	−18·3	+13·8	+16·6 (red)
	60–120	−14·7	+20·0	+17·5	−15·7	+14·0	+13·5 (v. pale pink)
	120–180	−13·1	+15·5	+15·9	−15·1	+13·8	+13·7 (colourless, re-oxidized to red on admitting air)

* Not the iodide.

salt solution unlike that previously employed [Dickens, 1936, 1] which was therefore impure. In aerobic experiments with tissue the phenazonium salts become partly oxidized to a red compound, presumably this one. This compound inhibited anaerobic glycolysis more than phenazine methochloride, but did not reduce the aerobic glycolysis as much in Exp. 3 of Table IV, though it had a pyocyanine-like action on aerobic glycolysis.

Autoxidation. Measured in $NaHCO_3$-glucose Ringer solution, by the two-vessel method of Warburg under the same conditions as in the tissue experiments, the autoxidation of 10^{-3} *M* phenazine methochloride in 95 % O_2+5 % CO_2 was slow, -3μl. O_2, $+4\mu$l. CO_2 per 4 ml. per hr. When tissue is present the oxidation is probably greater, but it is difficult to allow for it accurately. Calculations showed that with the quantities used, it could not result in any serious error, and that the measurements were substantially those of tissue metabolism and not of autoxidation of the carrier. When in experiments without tissue, in which autoxidation was allowed to proceed in a more alkaline medium, the O_2 consumption was measured and the acid production was titrated, it was found that for 1 mol. phenazine methochloride 0·5 mol. O_2 is consumed and 1 equiv. acid (1 mol. HCl) is set free. Hence autoxidation would be expected to increase aerobic acid production, whereas the addition of phenazine methochloride to tissue slices produces a fall of aerobic glycolysis and it is in fact probable that part of the residual acid production in presence of phenazine methochloride arises from this. On the other hand reduction anaerobically may have given values for glycolysis which were a little too high, but calculation showed a max. error of *c.* 10 %.

4·6 mg. phenazine methochloride were added from the side bulb of a Warburg manometer vessel to 4 ml. *N*/100 NaOH contained in the main part. Air, 37·5°. The initially rapid oxidation became very slow after 50 min. when 199 μl. O_2 had been consumed and the equiv. of 1·80 ml. *N*/100 acid liberated = 403 μl. Calc. for $\frac{1}{2}O_2$ and 1HCl; 224 μl., 448 μl. respectively. A bright red $CHCl_3$-soluble compound resulted. Indophenoloxidase preparation from heart muscle brings about oxidation of phenazine methochloride at neutral reaction to form a red compound soluble in $CHCl_3$. The quantitative investigation of this has not been made. Free phenazine in addition to the red compound results from alkaline oxidation of the methochloride [McIlwain, 1937, 2].

Summary

The E_h of a series of phenazine derivatives, determined by potentiometric titration, have been correlated with the electronic properties of the substituents. The activity of the phenazines and some non-phenazine dyestuffs as carriers in the hexosemonophosphate system has been determined. According to their activity, the substances fall into four groups: (1) most active: phenazine methosulphate, methochloride and ethosulphate, E_h+0·055 to +0·080; (2) moderately active: mono- and di-sulphonic acids derived from *N*-methylphenazine, E_h+0·13, +0·23; pyocyanine, E_h−0·011 and its ethyl homologue; (3) less active: brilliant cresyl blue and methylene blue; (4) others inactive: the most active of the above compounds had only about 1/250th of the turnover no. of the flavin enzyme, E_h−0·06. Structure is evidently more important than E_h in determining the activity of carriers for this system. A property common to the phenazines and flavin is the ability to form semiquinones, and the peculiar activity of these compounds in this system may be due to this property.

When phenazine methochloride is used instead of flavin enzyme, the Neuberg ester is attacked much less readily than the Robison ester; with flavin enzyme as carrier both are attacked at about the same rate. Carboxylase is inhibited by *M*/300 phenazine methochloride, not by pyocyanine or phenosafranine; succinic

dehydrogenase is inhibited by phenosafranine, but much less by phenazine methochloride in the concentration used. The hexosemonophosphate system is inhibited by iodide in low concentration. The action of phenazine methochloride on the metabolism of tumour tissue is to increase respiration and diminish aerobic glycolysis.

We wish to thank Prof. O. Warburg for generous gifts of purified enzyme and coenzyme, and Prof. R. Robison for the analysis of our hexose monophosphate. We are much indebted to Dr B. C. J. G. Knight for help and advice in connexion with the E_h measurements.

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CCXI. OXIDATION OF PHOSPHOHEXONATE AND PENTOSE PHOSPHORIC ACIDS BY YEAST ENZYMES

I. OXIDATION OF PHOSPHOHEXONATE

II. OXIDATION OF PENTOSE PHOSPHORIC ACIDS

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In a preliminary note [Dickens, 1936] a scheme of oxidation of carbohydrate was outlined in which the first stages suggested were the esterification of the hexose to hexosemonophosphate, oxidation of this first to the phosphohexonate, then to the 2-ketophosphohexonate, decarboxylation to a pentose phosphoric acid and by a continuation of the process finally pyruvic acid, which would be completely combusted.

At that time the evidence in favour of this scheme was slight. No proof of the formation of pentose phosphoric acids by oxidation of hexosemonophosphate or of phosphohexonate existed. The theory of the formation of 2-ketophosphohexonic acid was first advanced by Lipmann [1936] on the basis of O_2 uptake experiments only. Lipmann found that phosphohexonic acid was oxidized by yeast macerate, and that for each mol. phosphohexonate $\frac{1}{2}O_2$ was consumed and 1–1·5 CO_2 appeared, the CO_2 being only about 1 mol. in presence of bromoacetate. On the basis of this evidence, Lipmann advanced the theory that 2-ketogluconic acid was first formed ($\frac{1}{2}O_2$ consumed), this was then decarboxylated ($1CO_2$ liberated) and the further process consisted in fermentation of the theoretically expected *d*-arabinosemonophosphate, this stage being the one inhibited by bromoacetate. Some evidence tending to show keto-acid formation was given by experiments [Dickens, 1936] in which HCN appeared to act as ketone fixative, and the addition of a preparation of carboxylase to a carboxylase-free enzyme preparation caused a further oxidation and CO_2 evolution with phosphohexonate.

None of these results, however, was sufficiently clear to provide a basis for this theory. Warburg & Christian [1936, 1] showed, by using a more active enzyme preparation, that the "end-point" in Lipmann's experiments was only apparent and was in reality due to a gradual destruction of the activity of the enzyme. The experiments with HCN and carboxylase [Dickens, 1936] were too indirect. The critical points which had to be shown were:

1. Pentose phosphoric acids must be shown to arise from the oxidation and decarboxylation of hexose phosphoric acids.

2. It must be shown that pentose phosphoric acids are readily oxidized and fermented by yeast extracts.

Both these points are established in the work to be described. In the first part of this paper the oxidation of phosphohexonic acid is described, and confirmation of the occurrence of 5-C phosphoric esters giving the pentose reaction [Warburg & Christian, 1937] is obtained. Further oxidation of the phosphohexonic acid to a

4-C phosphoric ester is shown to occur. In the second part, it is shown that pentose phosphoric acid can be readily oxidized by yeast enzyme in the presence of coenzyme II.

In the subsequent paper [Dickens, 1938] it will be shown that pentose phosphoric acid is readily fermentable by yeast enzymes in the presence of coenzyme I and inorganic phosphate.

During this work, the important point arose that the pentose phosphoric acid most readily attacked both oxidatively and by fermentation, is not the *d*-arabinose-5-phosphoric acid which would be expected to arise from simple 2-oxidation and decarboxylation of *d*-glucose, but is the stereoisomeride *d*-ribose-5-phosphoric acid. Since *d*-ribose is the naturally occurring pentose of animal and yeast cells, this fact is of great physiological interest, and may perhaps give a key to the metabolism of pentoses in living cells. *d*-Ribose is not itself fermentable, either by yeast cells or yeast extract, nor is it oxidized by brain slices or by yeast extract. Apparently direct phosphorylation of ribose presents difficulty in these materials, and the theory is advanced that *d*-ribose-5-phosphoric acid may originate from hexoses by a process of phosphorylation, oxidation and decarboxylation. Indirect evidence is presented in these papers which makes this assumption probable, and direct evidence will be sought in future experiments.

I. OXIDATION OF PHOSPHOHEXONATE

Enzyme. Prep. A. The enzyme preparation used in most experiments was made from Lebedew yeast maceration fluid by the method of acetate precipitation already described [Dickens & McIlwain, 1938] and the general methods for experiment were the same as detailed there, except that phosphohexonate was used as substrate. During the course of this work we have used three different yeasts: (1) Newcastle Brewery Co. top fermentation yeast. (2) Messrs Tennent, Glasgow, bottom yeast. (3) Löwenbräu, Munich, bottom lager beer yeast. Although there was little difference in the activities of the macerates from these, successful precipitation of enzyme without excessive dilution of the Lebedew juice was obtained only with the German yeast, presumably owing to its lower content of protective hop resins, which held up the precipitate with the British yeasts.

Prep. B. In earlier experiments a preparation was made from Tennent's yeast Lebedew juice by diluting 50 times with distilled water, passing CO_2, centrifuging from the inactive precipitate after 10 min., passing CO_2 to saturation and keeping in a stoppered bottle in the refrigerator overnight [cf. Warburg & Christian, 1933]. The centrifuged precipitate, dried *in vacuo* over H_2SO_4 and KOH was used: yield *c.* 250 mg. per 100 g. dried yeast; 5–20 mg./vessel were needed. This preparation attacked hexosemonophosphate more rapidly than phosphohexonate; otherwise there was little difference between A and B, but both differ from "Proteins I and II" of Warburg & Christian [1937] in that the precipitated enzyme is washed with acid in our two preparations, and this may account for some differences between the results of Warburg & Christian and those to be described.

The activities of the original Lebedew juice and of Preps. A and B are shown in Table I. It is evident that much activity is lost in the precipitation, and this passes into the acid supernatant liquid, from which Warburg & Christian [1937] prepare their enzyme. This loss is compensated for to some extent by the simplicity of the method and by the greater stability of the acid-precipitated enzyme.

Table I. *Activities of original Lebedew juice and Preps. A and B*

All experiments at 38°, O_2, 0·2 ml. KOH in inner cup.

Original Lebedew juice. Diluted to 2·2 ml. with *M*/100 phosphate containing 0·1 mg. coenz. II, 0·3 ml. *M*/10 substrate, and 0·2 ml. *M*/100 phenazine methochloride.

Source	Per vessel ml.	μl. O_2 consumed per hr. with substrate		
			Hexosemono-phosphate	Phospho-hexonate
Newcastle Brewery Co.	0·20	34	226	287
	0·05	4	56	63
Löwenbräu, Munich	0·20	—	225	311
	0·05	—	63·5	141

Acetate precipitates (A). Dissolved in *M*/50 phosphate of *p*H 7·4, *p*H *c.* 6·5. 0·1 mg. coenz. II; 0·3 ml. *M*/10 substrate; 0·2 ml. *M*/100 phenazine methochloride.

Source	Mg.	Corrected for blank (*c.* 10 μl./hr.)	
Löwenbräu, Munich	5	55	50
	10	90	90
	20	121	141
	50	155	180
CO_2 precipitate (B):			
Tennent, Glasgow	5	207	42

Numerous attempts to prepare stable extracts from the acid supernatant liquid proved unsuccessful: Warburg & Christian [1937] have since overcome this difficulty by warming the $\frac{1}{2}$-sat. ammonium sulphate solution of the enzyme to 50°, thus "stabilizing" it, but the work described in this section was done before the publication of this method. Presumably destructive enzymes are removed by both methods.

Phosphohexonate. In a few earlier experiments, but not in any of the isolation experiments, the phosphohexonate was made from the prep. of muscle hexosemonophosphate described in the preceding paper [Dickens & McIlwain, 1938] by the method of Robison & King [1931]. Later Prof. Robison kindly gave a prep. of the neutral Ba salt of 6-phosphogluconate containing some 6-phosphomannonate with $<1{\cdot}5\,\%$ of reducing hexosephosphate. Analysis (Prof. Robison): H_2O, 7·0 %. Found (dry subs.): total P, 6·34 %; inorg. P, 0·06 %; H. and J. titn. with added NaOH, 0·5; $[\alpha]_{5461}-1{\cdot}2°$. Calc. for $(C_6H_{10}O_{10}P)_2Ba$: P, 6·42 %.

The solution of Na salt was prepared by grinding the Ba salt (288 mg.) with a slight excess of Na_2SO_4, $10H_2O$ (295 mg.) and centrifuging from Ba_2SO_4 after making up to 6 ml. with water and addition of 0·055 ml. *N* HCl. The concentration of ester P was estimated on this solution (*c.* 0·09 *M*).

Coenzyme. Coenzyme II of purity 1 was given by Prof. Warburg. Coenzyme II of purity 0·2 was prepared from horse blood corpuscles [Warburg & Christian, 1936, 2] and its activity checked by comparison with the Warburg preparation: yield 80 mg. from 2·2 l. washed corpuscles. This preparation contains also coenzyme I and an adenine nucleotide [Warburg & Christian, 1936, 2]. Usually 0·02 mg. of purity 1, or 0·1 mg. of purity 0·2 was used per vessel. Unlike the hexosemonophosphate oxidation, that of phosphohexonate is not always maximal with these amounts, and in some "end-point" experiments they were increased fourfold. Possible reasons for this are indicated later.

Carriers. Flavin enzyme and phenazine methochloride were used as previously [Dickens & McIlwain, 1938]. The O_2 uptake with phenazine methochloride was corrected for blank without substrate (autoxidation of phenazine methochloride + blank oxidation). The blank was small with Preps. A and B except in

a few "end-point" experiments where high concentrations of phenazine methochloride were used to ensure a rapid reaction. With flavin enzyme the blank was usually negligible.

Measurement of O_2 uptake. Warburg manometers filled with O_2 and containing KOH in the inner cup were used at 37·5°. Where HCN was present it was added as freshly prepared neutral solution, and instead of KOH, KCN-KOH mixture [Krebs, 1935] was used to avoid absorption of HCN. CO_2 evolved was measured (in absence of HCN) by a second vessel without KOH, which was acidified at the end of the exp. to drive out bound CO_2, with a similar pair of vessels without substrate as controls.

pH-activity curve. The titration curve of the enzyme (Prep. A, 20 mg./ml. in *M*/100 phosphate buffer) was first determined and the contents of each vessel after cooling in ice were adjusted to the required *p*H by addition of the required vol. ice cold *N*/100 NaOH or, for the vessel at *p*H 4·7, *N*/50 acetic acid. Stronger phosphate buffer cannot be used since it inhibits the reaction (see below). The enzyme system with phenazine methochloride as carrier showed a broad *p*H optimum at *p*H 6·3–7·5 (Fig. 1).

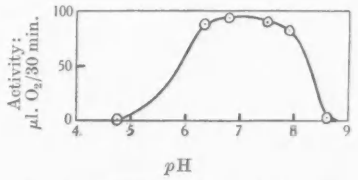

Fig. 1. *p*H optimum of phosphohexonic system.

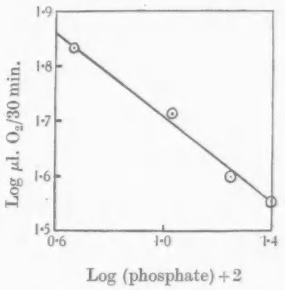

Fig. 2. Phosphate inhibition of phosphohexonic system.

Phosphate inhibition. Conditions were as in the preceding exp. except that *p*H was kept constant and varying concentrations of phosphate buffer were used. During the first 30 min. of the exp. the inhibitions with 0·05, 0·1 and 0·25 *M* phosphate were respectively 16, 32 and 57 %. Fig. 2 shows that, as with the hexosemonophosphate system [Theorell, 1935], the velocity of oxidation varies inversely as the phosphate concentration. Comparison with the data of Theorell shows less inhibition for a given phosphate concentration with the phosphohexonic system than with the hexosemonophosphate one. This also is shown in Fig. 1 of the preceding paper [Dickens & McIlwain, 1938].

Attempted separation of the phosphohexonic and hexosemonophosphate systems. The above behaviour towards phosphate inhibition is typical of the slight differences between these two systems, which makes their separation difficult. As yet attempts to obtain an enzyme which attacks only phosphohexonate have been uniformly unsuccessful, whether by dialysis, ammonium sulphate precipitation, acetone precipitation or partial inactivation by incubation with alkali. On the other hand, the "Zwischenferment" of Negelein & Gerischer [1936] is free from the phosphohexonic enzyme [Dickens, 1936; Warburg & Christian, 1937]. It is probable that our enzymes, like those of Warburg & Christian [1937], are mixtures of several components, as is shown by the different substrates attacked and the multiple products formed.

Table II shows that the acid-precipitated enzymes require coenzyme II for their activity, purified cozymase being without effect. Gluconic acid is not oxidized unless phosphorylated, and the preparations contain no active carboxylase. An experiment with Warburg "Zwischenferment" is included to show its relative actions on hexosemonophosphate and phosphohexonate. In this exp. (Table II) the less pure sample of phosphohexonate was used, and the small O_2 uptake is probably due to traces of reducing hexosephosphate in the substrate.

Table II. *Action on various substrates: coenzyme required*

Enzyme in $M/100$ phosphate of pH 7·4; 0·04 mg. coenzyme II of purity 1; or 0·6 mg. purified cozymase; 0·5 ml. 0·8 N/HCN. Side bulb, 0·2 ml. 10% flavin enzyme. Inner cup, KCN-KOH. 0·4 ml. $M/10$ substrates. O_2. 37·5°.

Enzyme and coenzyme	Enzyme wt. per vessel mg.	Time min.	O_2 uptake (μl.) with substrate: Hexosemono-phosph.	Phospho-hexonate	Gluconic acid
Enz. B.:					
Coenz. I	8	30	—	− 2	0
		60	—	− 5	0
		90	—	− 6	—
Coenz. II	8	30	− 73·5	−49·5	−0·5
		60	−125	−78	−1
		90	−155	−101	—
Warburg "Zwischenferment":					
Coenz. II	0·1	30	−246	−13·5	—
		60	−313	−17·5	—

Enzyme B in $M/100$ KH_2PO_4, 0·2 ml. $M/5$ Na pyruvate, CO_2 evoln. (90 min.) + 2·5 μl.

Action of HCN. In the earlier experiments which were made with acid-precipitated enzyme and coenzyme II of purity 1, HCN (0·12 M) was necessary for the reaction to continue [Dickens, 1936]. This result (Fig. 3) was obtained many times when the pure coenzyme was used with small quantities of the enzymes A or B. By increasing the amount of enzyme, however, it was no longer apparent, the reaction continuing even in the absence of HCN. In the later work more care was taken to prevent destruction of enzyme during its precipitation and washing, by using ice cooling and quick manipulations, and it is possible that this may account for the difference between earlier and later preparations in their need for HCN. We are unable to account for the results otherwise.

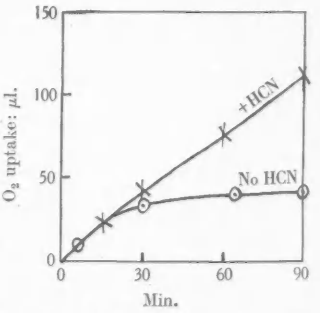

Fig. 3. Effect of HCN on O_2 uptake with phosphohexonate (earlier prep. of enzyme).

End-point with pure coenzyme II. Table III shows experiments with and without HCN for both hexosemonophosphate and phosphohexonate. In all these, coenzyme II of purity 1 was used; different end-points obtained with cruder coenzyme are described later.

The results of Table III show that under the conditions used, i.e. with pure coenzyme and vigorous enzyme preparations, the reaction proceeds either in presence or absence of HCN to an end-point which with phosphohexonate is

Table III. *End-point with pure coenzyme and acid-precipitated enzyme*

No.	Phosphohexonate or hexosemono-phosphate ml. 0·09 *M*	μl.*	Enzyme prep. type	μl. O_2 uptake after hr. ½	1	2	3	4	5	6	End-point. % of 1 mol. O_2 per mol. substrate
With HCN (0·12 *M*): Phosphohexonate											
1	0·4	810	A	130	222	329	360	380	—	390	48
2	0·2	402	B	56·5	122	181·5	—	—	—	—	45
3	0·1	202	B	56	77	85	87·5	—	—	—	43
4	0·1	202	B	33	64	99·5	104	—	104	—	51
5	0·05	101	A	35	54	57·5	56	—	—	—	51
Hexosemonophosphate											
6	0·1	202	B	51	89	154	166	172	—	—	85
7	0·05	101	A	33	62	87·5	92	—	—	—	90
Without HCN: Phosphohexonate											
8	3·0	6050	A	—	—	—	—	—	—	3115	51·4
9	0·2	401	B	40·5	81·5	136·5	—	157	—	167	42
10	0·1	202	B	37	61	78	98	107	—	—	53
11	0·05	101	B	31	43	53·5	64	66	—	—	65
Hexosemonophosphate											
12	0·1	202	B	51	89	166	172	—	—	—	85
13	0·05	101	B	—	87·5	101	117	—	—	—	115

* 1 m*M*. = 22400 μl.

close to that required for $\frac{1}{2}O_2$ per mol., with hexosemonophosphate $1O_2$ per mol. substrate. Although some very slow residual oxidation may remain, the figures of Table III, and still better the curves of these experiments, only one of which (Fig. 4) is reproduced, show the definite change in reaction rate at these points.

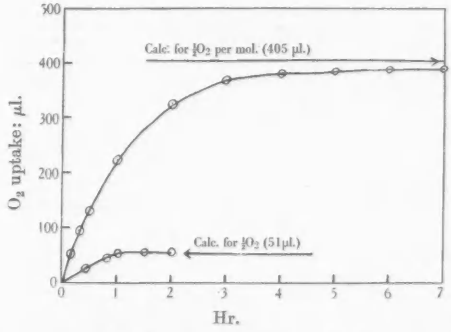

Fig. 4. End-point of phosphohexonic oxidation with coenzyme II of purity 1.

It seems most unlikely that this could be due in every case to destruction of enzyme coinciding with the different reaction times. Exp. No. 2, Table III, in which the enzyme with substrate and carrier, but without coenzyme, was incubated in the vessel for 1½ hr. at 37·5° before the coenzyme was tipped in, still gave a similar end-point corresponding to 90% of the O_2 uptake required for $\frac{1}{2}O_2$ per mol. phosphohexonate.

The end-point observed corresponds fairly closely with the oxidation of both substrates to a stage corresponding with the formation of ketophosphohexonate.

H_2O_2 formation. H_2O_2 results from the autoxidation of the flavin enzyme when hexosemonophosphate is oxidized by the purified enzyme [Warburg & Christian, 1933]. H_2O_2 did not accumulate in the present experiments since (1) the acetate-precipitated enzyme contained very active catalase (25 mg. enzyme, 0·2 ml. 0·3 % H_2O_2, 114 μl. O_2/5 min.) (2) HCN did not affect the end-value although it would have inhibited the catalase. Accumulation of H_2O_2 would have changed the interpretation of the end-point, but the above experiments show that it did not occur.

Other ketone fixatives. The action of HCN which was seen with the earlier enzyme preparations, might have been due to its action as ketone-fixative, as in the experiments of Green & Brosteaux [1936] with lactic dehydrogenase. As these authors found for the lactic system, semicarbazide (M/20) and hydrazine hydrate (M/8) were without effect in the phosphohexonic system. Hydroxylamine and phenylhydrazine (M/8) caused irregular pressures; with sulphite and bisulphite no combination with keto-substance could be demonstrated by I_2 titration. Increases of O_2 uptake and CO_2 output equal to the O_2 consumed were regularly observed [Dickens, 1936] on the addition of crude carboxylase [Axmacher & Bergstermann, 1934], but this may be attributable perhaps to other enzymes or coenzymes contained in the carboxylase and should be repeated now that purified enzyme and cocarboxylase [Lohmann & Schuster, 1937] are available. Unphosphorylated 2-keto-*d*-gluconic acid is attacked only very slowly by active carboxylase (Table IV), but the phosphorylated compound may of course behave differently.

Table IV. *Action of yeast carboxylase*

50 mg. carboxylase prep., in 2 ml. M/50 phosphate buffer. Side bulb 0·1 ml. 2·5 N HCl. Air. 37·5°.

	μl. CO_2 evolved at			
	*p*H 7·4 Addition: 0·2 ml. M/10 Na salt		*p*H 6	
Min.	Pyruvate	2-Ketogluconate	Pyruvate	2-Ketogluconate
5	+ 30	0	+228	0
30	+152	0	+363	+5
60	+173	0	+391	+7·5
120	—	—	—	+9

Product from the oxidation of phosphohexonate with $\frac{1}{2}O_2$. A larger scale exp. was made to determine the nature of the product from the oxidation of phosphohexonate in presence of pure coenzyme II.

195 mg. enzyme A, well washed with acetate buffer [Dickens & McIlwain, 1938], were dissolved in 2·2 ml. water and the *p*H adjusted to 7·3 with ice cold N/100 NaOH (7·3 ml.). 0·3 mg. coenzyme II of purity 1 and 3 ml. 0·09 M Na phosphohexonate were added, the mixture was placed in two large Warburg manometer vessels containing 1·5 mg. phenazine methochloride and 0·5 ml. KOH in the side bulb and inner well of each vessel. O_2, 37·5°.

After 5 hr. the reaction became very slow and 3680 μl. O_2, corr. for autoxidation of the carrier, had been consumed. The oxidation thus went a little beyond the consumption (calc. 3050 μl.) of $\frac{1}{2}$ mol. O_2 per mol. phosphohexonate, perhaps because of traces of coenzyme contained in the large amount of enzyme used. The contents were precipitated with trichloroacetic acid and the supernatant liquid was neutralized with $Ba(OH)_2$, the resulting precipitate, together with that from addition to the mother liquor of 2 vol. abs. alc. was dried *in vacuo*; wt. 149 mg. This was placed in 5 ml. water, with N/10 HCl to make weakly acid. After centrifuging from the undissolved residue, the clear solution was neutralized with $Ba(OH)_2$ and was poured into 2 vol.

abs. alc. The precipitate ("Ba salt I") was well washed with 75% and abs. alc., then with ether and was dried *in vacuo* over P_2O_5; wt. 91 mg. =60% of the amount of Ba phosphohexonate taken. Analysis (Weiler): Dried at 60° *in vacuo*, loss of wt. 7·26%. Found, for dry subs.: C, 15·50; H, 2·23; P (as phosphomolybdate) 7·04; Ba, 37·1%. Calc. for $C_5H_9O_9PBa$: C, 15·75; H, 2·36; P, 8·13; Ba, 36·1%. Calc. for $C_6H_{10}O_{11}PBa_{1\cdot25}$: C, 15·62; H, 2·18; P, 6·73; Ba, 37·20%. The second formula is that of an acid Ba salt of 2-ketophosphohexonic acid; the first is that of the carboxylic acid derived from 2-ketophosphohexonic acid by oxidative decarboxylation, i.e. a phosphopentonic acid.

It seemed very probable that Ba salt I might be a mixture of these two compounds. Unfortunately their separation could not be achieved on the specimen available, and the repetition of the exp. has been left for a future occasion. An attempted purification by dissolving in HCl and making more alkaline than previously with $Ba(OH)_2$, resulted in a Ba salt containing much less P and Ba, these elements now being in the ratio 0·96:1. Found (Weiler): loss of wt. at 60° *in vacuo*, 5·02%. Dry subs. P, 5·23%; Ba, 24·4%. Some decomposition appeared to have occurred and the mixture could not be separated. Solution of a sample of the original Ba phosphohexonate in HCl and its reprecipitation by $Ba(OH)_2$ at similar alkalinity caused no such decomposition.

Oxidation of phosphohexonate in presence of coenzyme of purity 0·2: end-point and CO_2 formation. In the presence of the less pure coenzyme the oxidation with enzyme preps. A and B proceeds further than in the preceding experiments. Provided that sufficiently active enzyme is used in the presence of plenty of coenzyme, the O_2 uptake slightly exceeds 1 mol. O_2 per mol. phosphohexonate

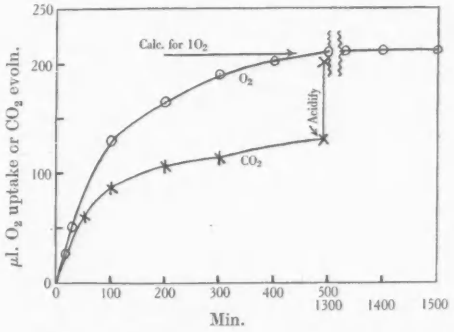

Fig. 5. End-point of phosphohexonic oxidation with coenzyme II of purity 0·2.

(Fig. 5) and the CO_2 formed is almost exactly equivalent to the O_2 uptake when the reaction has gone to completion. If however the reaction is stopped by acidification before the end-point is reached, the CO_2 formed then exceeds the O_2 consumed (Table V).

These results seem to indicate the occurrence during the earlier stages of the oxidation of an excess of decarboxylation over O_2 uptake. This could occur in two different ways: (1) initial stage; $\frac{1}{2}O_2$ consumed, keto-acid formed, keto-acid decarboxylated forming pentose phosphoric acid with evolution of 1 mol. CO_2; later slower oxidation by a similar type of mechanism of the pentose phosphoric acid formed. (2) Pentose phosphoric acid formed as in (1), but then undergoing cleavage with oxidation of the cleavage products formed.

Table V

Enzyme: 20 mg. per vessel Prep. A dissolved in 1·5 ml. *M*/50 phosphate buffer at *p*H 7·4, initial *p*H of mixture 6·22; 0·2 ml. = 0·1 mg. coenzyme of purity 0·2; 0·2 ml. *M*/100 phenazine methochloride in main part of vessel. Temp. 37·5°. O_2. Amount of substrate (from P estimation) = 404 μl./vessel.

Vessel no. ...	1	2	3	4
Enzyme, coenz., carrier, ml.	1·9	1·9	1·9	1·9
Bulbs:				
I. *M*/10 phosphohex., ml.	0·2	0·2	No substrate	
II. 2·5 *N* HCl, ml.	—	0·1	—	0·1
Inner cup, ml.	0·2 KOH	—	0·2 KOH	—
μl. O_2 or (free) CO_2 after min.	O_2	CO_2	O_2	CO_2
20	− 67·5	+ 60·5	− 0·5	+ 0·5
40	− 101	+ 93	− 0·5	+ 0·5
60	− 160	+ 147·5	− 1·5	+ 0·5
120	− 237	+ 206	− 4·5	+ 2
Total CO_2 on acidification	—	+ 329 uncorr. + 310·5 corr. for No. 4	—	+ 18·5

Hence when 0·58 mol. O_2 per mol. phosphohexonate has been consumed, the ratio $CO_2/O_2 = 1·31$.

At present it is not possible to decide between these alternatives, but it will be shown that both oxidation and fermentation of pentosemonophosphate can occur, and that a 4-C monocarboxylic-monophosphoric acid is formed oxidatively under conditions similar to those in the above exp.

Nature of the products formed from the oxidation of phosphohexonate in presence of crude coenzyme

Warburg & Christian [1937] have investigated the products formed when phosphohexonate is oxidized by flavin enzyme, pure coenzyme II and a yeast enzyme (prepared without washing with acid: "Protein of protein fraction I, 4th step") in the presence of methyl alcohol. This preparation differs from ours in its action. With our enzyme, and with phenazine methochloride as carrier and coenzyme of purity 0·2, the oxidation of phosphohexonate becomes slow when 1 mol. O_2 is absorbed and 1 mol. CO_2 has been evolved. With the Warburg & Christian system, the reaction stops when 0·6 mol. O_2 has been consumed and 0·7–0·8 mol. CO_2 has been liberated.

In view of these differences, the products formed under our conditions were investigated. The fractionation of the products followed the description given by Warburg & Christian [1937, p. 293] exactly, and details of fractionation are therefore omitted.

1·86 g. enzyme prep. A was dissolved in 130 ml. water + 28 ml. ice cold *M*/50 NaOH. 120 mg. phenazine methochloride and 9·5 mg. coenzyme II (purity 0·2) were added, and the mixture was shaken at 37·5° in O_2 with 10·0 ml. 0·09 *M* phosphohexonate; *p*H of mixture 6·4. An aliquot part of the mixture was used to measure O_2 consumption, with a control using water instead of substrate, this exp. running concurrently.

Manometric readings: 1·5 ml. enzyme solution (containing the coenzyme and phenazine methochloride) in main part of vessels. 0·1 ml. 0·09 *M* phosphohexonate (or water) in side bulb. 0·2 ml. KOH in inner cup. O_2. 37·5°.

Vessel no.	Add at t_0	μl. O_2 consumed after min. 30	60	120	240	1100	1200
1	Phosphohex.	64	100	144	195	266·5	267·5
2	Water	6	9	11	16	25·5	26·5
Difference μl. O_2: (1–2)		58	91	133	179	241	241

Calc. for 0·1 ml. (*M*/10) × 0·91 phosphohexonate (P analysis): 204 μl. O_2 if 1 mol. phosphohexonate consumes 1 mol. O_2.

The trichloroacetic acid filtrate was worked up by Warburg & Christian's method. The filtrate contained 28·7 mg. combined P; amount of ester P added as phosphohexonate = 0·91 × 31·0 = 28·2 mg. Evidently little breakdown of organic P occurred. As in Warburg & Christian's exp., neutral and basic lead acetate yielded distinct precipitates of Pb salts; neutral (after removal of inorg. P): wt. 315 mg. Fractionated by Hg salt; Hg precipitable converted into Pb salt; wt. 85 mg. "A". Hg supernatant fluid reprecipitated with neutral Pb acetate; wt. 76 mg. "B". Basic Pb acetate precipitate from supernatant liquid of neutral Pb acetate precipitation, converted into Ba salt.; wt. 60 mg. "C". Salts "A" and "B" were redissolved and reprecipitated for analysis.

Analyses. (Weiler, except colorimetric P estimations on Pb but not on Ba salts. All figures are expressed on subs. dried at 40° *in vacuo* unless otherwise stated.)

Subs. "*A*". Found: Pb, 63·40; P, 6·26 %. Warburg & Christian's Pb salt 1, prepared similarly from the Hg precipitate, had Pb, 61·5; P, 6·2 %. The two were not identical however since Pb salt "A" gave the orcin test and contained more C (ratio C:P:Pb = 4·7:1·01:1·53). Consistent analyses were not obtained and the fraction was probably a mixture.

Subs. "*B*". Found: C, 8·92; H, 1·43; P, 5·60; Pb, 57·0 %. Calc. for $C_4H_6O_8PPb_{1\cdot5} + H_2O$: C, 8·86; H, 1·47; P, 5·71; Pb, 57·3 %. Subs. "B" dried *in vacuo* at 100°. Found: C, 9·47; H, 1·56 %. Calc. for $C_4H_6O_8PPb_{1\cdot5}$: C, 9·16; H, 1·15 %.

Subs. "B" thus corresponds closely with the monohydrated neutral Pb salt of a monophospho-erythronic or -threonic acid: $Pb_{\frac{1}{2}}OOC.(CH.OH).(CH.OH).CH_2O.PO_3Pb + H_2O$. In the corresponding fraction from their oxidation, Warburg & Christian [1937] obtained a Pb salt, $C_5H_8O_9PPb_{1\cdot5}$ containing (CH_2O) in excess of our Pb salt. Taken in conjunction with the results described here, this can be taken as strong presumptive evidence that this salt of Warburg & Christian was the next higher member, namely the Pb salt of the phosphopentonic acid. These facts, and the isolation of the pentose-containing fraction "C" described below, together provide strong experimental support for the existence of a system of progressive oxidative breakdowns of the sugar-monophosphoric acids, such as was previously tentatively suggested [Dickens, 1936].

It is particularly interesting that in our series, where the oxidation proceeded further, the O_2 uptake being 1 mol. per mol. substrate, instead of 0·5 mol. as in Warburg & Christian's exp., the degradation of the C-chain has proceeded one stage further. As a variety of stages of oxidation are simultaneously present, it is not possible to correlate the O_2 uptake more than roughly with the extent of breakdown. But it is noteworthy that the formation of fraction "B" in our exp. required the consumption of only half the amount of O_2 (2 mol.) calc. from the following equations:

COOH	COOH	CO_2				
CH.OH	CO	+ CHO	COOH	COOH	CO_2	COOH
$CH.OH + \frac{1}{2}O_2 =$	$CH.OH =$	$CH.OH + \frac{1}{2}O_2 =$	$CH.OH + \frac{1}{2}O_2 =$	$CO =$	$CHO + \frac{1}{2}O_2 =$	$CH.OH =$
CH.OH	CH.OH	CH.OH	CH.OH	CHOH	CH.OH	CH.OH
CH.OH	CH.OH	CH.OH	CH.OH	CH.OH	CH.OH	$CH_2.O.PO_3H_2$
$CH_2.O.PO_3H_2$	$CH_2.O.PO_3H_2$	$CH_2.O.PO_3H_2$	$CH_2.O.PO_3H_2$	$CH_2.O.PO_3H_2$	$CH_2.O.PO_3H_2$	
Phospho-hexonate	(?) Ba salt I (Dickens)		(?) Pb salt I (Warburg & Christian)			(?) Pb salt "B" (Dickens)

Similarly for the formation of Warburg & Christian's Pb salt 1, the consumption of 1 mol. O_2 would be required, whereas only half this amount was observed as the total O_2 uptake in their exp. (The CO_2 outputs in the two series of exp. were also about half those calc. from the above equations.) Since the calc. O_2 uptakes (and CO_2 outputs) were in excess of the observed amounts, it is evident that the excess oxidation of this stage must be accompanied by the formation of less highly oxidized products. That this was in fact the case in our exp. is shown by the analysis of Ba salt "C".

Subs. "*C*". Found: C, 23·72; H, 3·02; P, 5·21; Ba, 24·80. Calc. for $C_{11}H_{21}O_{15}PBa$: C, 23·50; H, 3·74; P, 5·52; Ba, 24·50 %. This formula corresponds empirically with the Ba salt of a compound containing 1 mol. hexose, 1 mol. pentose and 1 mol. H_3PO_4, with the addition of 1 atom O and the loss of 1 mol. H_2O. Since it gives an intense pentose reaction (see below) it is apparently a combination of 1 mol. pentose with 1 mol. phosphohexonic acid, or alternatively of 1 mol. pentose phosphoric acid with 1 mol. hexonic acid. The fate of the lost phosphoric acid mol. is unknown: since the inorganic P did not appreciably increase during the exp., either H_3PO_4 was lost during the isolation, or else was used during the incubation to phosphorylate another molecule.

Pentose reaction. The orcin-$FeCl_3$ test was used [Tollens-Elsner, 1935, p. 111]. The phosphohexonate used gave a negative reaction, or at most the faintest trace of green which developed only very slowly (2-keto-*d*-gluconate behaved similarly). Pb salt "A" and Ba salt "C" gave intensely positive reactions (*c.* 1 mg. used for test); pigment sol. in amyl alcohol with absorption bands at 595–600 and 650–60 mμ. As was to be expected from its analysis, Pb salt "B" gave no colour with the pentose test. Unfortunately the quantities were insufficient for quantitative pentose estimation; the colours in the micro-method of McCance [1926] were found to fade rapidly with pentose phosphoric acid (ribosephosphate) and to differ too much from one pentose to another.

II. OXIDATION OF PENTOSE PHOSPHORIC ACIDS

Since in the preceding part it has been shown that pentose phosphoric acids or compounds closely resembling these are formed in the oxidation of phosphohexonate by yeast enzymes, and that compounds representing still further oxidation stages are also present, particularly the substance analysing as a phosphoerythronic acid, the next step was evidently to study the oxidation of those pentose phosphoric acids that are available.

I am greatly indebted to Dr P. A. Levene, who has added so much to knowledge of pentose phosphoric acids, for generous gifts of synthetic esters, without which these experiments would have been very incomplete.

Pentose phosphoric acids

d-*Ribose-5-phosphoric acid.* (*a*) *Natural.* Ba inosinate (Laoköon, Lwów) was hydrolysed and the Ba salt isolated by the method of Levene & Jacobs [1911]. Yield 2·9 g. from 5 g. Ba salt. Recryst. from 10 ml. hot water (animal charcoal) with addition of 10 ml. alcohol to the mother liquor: yield 1·7 g. The white crystalline subs. was dried *in vacuo* over H_2SO_4 for 24 hr. Analysis (Weiler), dried at 100° *in vacuo* loss of wt. 8·64 %. Found (dry subs.): Ba, 37·7; P, 8·88 %. Calc. for $C_5H_9O_8PBa$: Ba, 37·6; P, 8·49 %: $+2H_2O$ req. 8·97 %.

(*b*) *Synthetic.* Dr Levene's prep. of the synthetic Ba salt [Levene & Stiller, 1934].

d-*Arabinose-5-phosphoric acid Ba salt* (synthetic). Found (Dr Levene): Ba, 37·3; P, 8·77 %. *Xylose-5-phosphoric acid* (*Ba salt*). Synthetic, from Dr Levene [Levene & Raymond, 1934]. 2-*Keto*-d-*gluconic acid* (*Ba salt*). Prepared according to Ohle & Koller [1924].

Preparation of c. *M/10 solutions of Na salts.* These were prepared freshly, as on keeping in the ice box the solutions turned yellow. 77 mg. Ba salt ground with 65 mg. Na_2SO_4 $10H_2O$ were made up to 2 ml. with addition of 0·02 ml. *N* HCl. Found (ribose phosphoric acid): organic P, 0·756, 0·765 *M*/10.

The same enzyme preparations, carriers and coenzymes were used as in the preceding section, unless otherwise stated, and the experimental procedure was the same.

Oxidation experiments

1. *Oxidation by Lebedew extract.* For these experiments yeast macerate, either undiluted or with *M*/100 phosphate buffer, *p*H 7·4, *p*H of mixture *c.* 6·4, was placed in the main part of Warburg manometers, containing in the side bulb 0·3 *M*/10 substrate (Na salts of acids), and in the inner cup 0·2 ml. KOH. O_2. 37·5°. In those exp. with extra carrier and coenzyme, 0·2 ml. *M*/100 phenazine methochloride and 0·1 mg. coenzyme II of purity 0·2 were added to the main contents.

Table VI. *Oxidation in Lebedew fluid*

Dilution with *M*/100 phosphate buffer of *p*H 7·4; *p*H of mixt. *c.* 6·5. 0·3 ml. *M*/10 substrates. KOH in inner cup. O_2. 37·5°.

No carrier or coenzyme added:

Source	Vol. per vessel ml.	μl. O_2 uptake in 1st hr. with substrate: None (water)	Glucose	Gluconic acid	2-Keto-gluconic acid	Phospho-hexonic	Ribose-5-phosph. acid
Löwenbräu:							
(Fresh)	1	40	196	116	44	212	126
(Overnight at 0°)	1	40	137·5	—	—	—	163
,,	0·2	11·5	68	—	—	—	51·5

With carrier and coenzyme added:

0·2 ml. Newcastle Brewery Lebedew fluid diluted to 2·2 ml. total vol. with *M*/100 phosphate; this vol. contains 0·2 ml. *M*/100 phenazine methochloride, 0·1 mg. coenz. II of purity 0·2; 0·3 ml. *M*/10 substrate. O_2. 37·5°. KOH in inner cup.

μl. O_2 in 1st hr. with: Water	Phospho-hexonic acid	Hexose mono-phosphoric acid	Xylose-5-phosphoric acid	*d*-Arabinose-5-phosphoric acid	*d*-Ribose-5-phosphoric acid	2-Keto-gluconic acid
42·5	186	—	—	46·5	146	—
29	—	205	27	34	108 (synthetic) 92 (natural)	32

Table VI shows that ribosephosphate is oxidized with both specimens of Lebedew fluid, whether diluted or undiluted, with or without the addition of the extra carrier and coenzyme. Lebedew fluid kept overnight in the ice box still oxidizes vigorously. Natural and synthetic ribosephosphates are both oxidized, but xylosephosphate and arabinosephosphate are little or not at all affected when phenazine methochloride is the main carrier present. The figures for keto-gluconic acid are included because of the suggestion that phosphoketogluconic

acid may be an intermediate in hexose oxidation. The unphosphorylated compound was not appreciably oxidized. Figures for phosphohexonate and hexosemonophosphate are included for comparison.

Fig. 6 shows the course of the reaction and the comparison between natural and synthetic ribose esters.

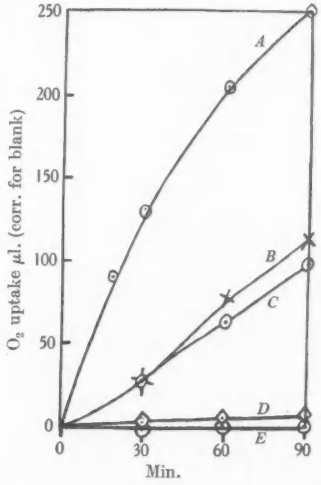

Fig. 6. Oxidation by Lebedew fluid (Newcastle Breweries). 0·3 ml. *M*/10 substrate +0·2 ml. Lebedew fluid + phenazine methochloride +0·1 mg. coenzyme to 2·2 ml. with *M*/100 phosphate. O_2. 37·5°. *A*: hexosemonophosphate. *B*: ribosephosphate (synthetic). *C*: ribosephosphate (natural). *D*: 2-ketogluconic *and* *d*-arabinose phosphate. *E*: *d*-gluconic acid.

2. *Boiled enzyme.* Table VII shows that the activity of Lebedew fluid towards ribosemonophosphate is destroyed by boiling.

Table VII. *Boiled and unboiled Lebedew fluid*

0·3 ml. Löwenbräu Lebedew fluid diluted to 1·5 ml. with *M*/30 phosphate of *p*H 7·4; *p*H of mixt. *c.* 6·5. 1·5 ml. per vessel either untreated or placed in a boiling water bath for 10 min. 0·3 ml. *M*/10 ribose phosphate or 0·3 ml. water. O_2. 37·5°.

	μl. O_2 uptake in 1st hr.			
	Ribosephosphate		Water	
Enzyme ...	Unboiled	Boiled	Unboiled	Boiled
	68·5	16	23	16·5

3. *Acetate precipitate from Lebedew fluid.* The enzyme used in the previous section (prep. A) is not suitable for the study of the oxidation of pentose phosphoric acids, since its activity is low and variable with this system at any rate when phenazine methochloride is used as carrier (Table VIII). That the preparation used was fully active towards hexosemonophosphate or phosphohexonate is shown by the control exp. with these substrates. Evidently different systems are concerned in the oxidation of pentose and hexose phosphoric acids. This explains the fact that the pentose esters were only in part further oxidized in our previous experiments with phosphohexonate.

Table VIII. *Acetate precipitate A*

20 mg. enzyme dissolved in 1 ml. $M/30$ phosphate buffer of *p*H 7·4; *p*H 6·4: 0·2 ml. $M/100$ phenazine methochloride; 0·1 mg. coenzyme I of purity 0·2. 0·2 or 0·4 ml. $M/10$ substrate. O_2. 37·5°. 0·2 KOH in inner cup.

		μl. O_2 uptake						
			Ribose-5-phosph.		Arabinose-5-phosph. (synthetic)		Hexose-monophosph.	Phosphohexonate
	Min.	Water	Synthetic 0·2 ml.	Natural 0·2 ml.	0·2 ml.	0·4 ml.	0·2 ml.	0·2 ml.
2 months' old enzyme	60	4·5	7·5	13·5	—	—	147·5	—
Freshly prepared enzyme	60	0·5	—	36·5	5	4·5	—	135
	120	1·0	—	64	12·5	18	—	214
	180	1·5	—	77	16	23	—	256

4. *Dialysed Lebedew fluid.* Lebedew fluid was dialysed for various times in a "Visking" cellophane tube against running tap water in a shaking machine. The results are shown in Table IX.

Table IX. *Dialysed Lebedew fluid*

1 ml. dialysed Lebedew fluid; 0·1 mg. coenzyme II of purity 0·2, or 0·2 mg. purified cozymase; 0·3 $M/10$ ribose-5-phosphoric acid or 0·3 ml. water. O_2. 37·5°. 0·2 ml. KOH in inner cup.

		μl. O_2 uptake						
		Water			Ribose phosphoric acid			
Dialysis hr.	Exp. min.	Water	Coenz. I	Coenz. II	Water	Coenz. I	Coenz. II	Coenz. I + II
4	60	20·5	—	25	110	—	146	—
16	60	0·5	—	2	6	—	38	—
	100	1	—	2	10	—	62·5	—
16	60	—	0	—	1	4·5	53	50
	120	—	0	—	5	10	109	116

4 hr. dialysis do not sufficiently remove the coenzymes, although an increase is now detectable on addition of coenzyme II (purity 0·2 throughout this work). On the other hand after 16 hr. the oxidation of ribosephosphate is very small unless coenzyme II is added, when it is about the same as in the original Lebedew fluid (Table I) after subtraction of the corresponding blanks without substrate. The blank is now very small in the dialysed Lebedew fluid. Cozymase (tested for activity with the lactic dehydrogenase) caused only a trifling increase, which may well be due to a trace of coenzyme II still contained in the purified cozymase. Addition of cozymase and coenzyme II together did not give any appreciable increase over coenzyme II alone. (Note however that the coenzyme II employed in these experiments certainly contained some cozymase.)

5. *Dried enzyme from dialysed Lebedew fluid. Preparation.* 150 g. Löwenbräu, Munich, dried yeast was macerated with 450 ml. distilled water for $2\frac{1}{2}$ hr. at 35°. The centrifuged Lebedew fluid (190 ml.) was dialysed as described above for 16 hr. against running tap water with shaking. After centrifuging from a considerable deposit, the supernatant, almost clear liquid was evaporated over H_2SO_4 in flat dishes in large vacuum desiccators evacuated with a Hyvac pump until the contents froze. The residue became almost dry in a few hr. The dry, light coloured, feathery powder weighed 6·2 g. For use, 80 mg. were taken in 1 ml. (equiv. 2 ml. original Lebedew fluid) distilled water per vessel. No carrier was added in these experiments, only substrate and 0·2 mg. coenzyme II being added. The solution

was centrifuged from a little denatured protein before use. The preparation retains it activity for long periods if kept cold and dry.

Activity of the dried preparation. Fig. 7 shows the activity with ribosephosphate, and that in the absence of coenzyme no significant O_2 uptake occurs. Since the O_2 consumed in Fig. 7 (0·1 ml. 0·076 M = 167 μl. ribose) exceeds

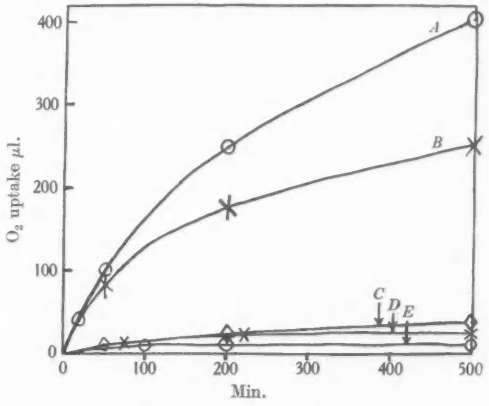

Fig. 7. *A*: 0·3 ml. 0·076 *M* ribosephosphate. *B*: 0·1 ml. ribosephosphate. *C*: 0·3 ml. 0·076 *M* ribosephosphate, no coenzyme. *D*: no substrate, with coenzyme. *E*: no substrate, no coenzyme.

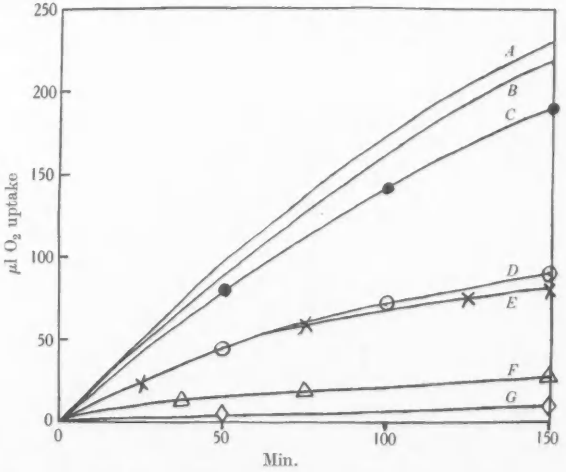

Fig. 8. Oxidation of various substrates (0·3 ml. $M/10$) by dried dialysed Lebedew fluid. *A*: phosphohexonic. *B*: hexosemonophosphate. *C*: ribosephosph. (Synthetic). *D*: *d*-arabinose phosph. *E*: xylosephosph. *F*: blank without substrate, with coenz. *G*: blank: no coenz., no substrate.

1 mol. O_2 per mol. ribosephosphate, it is evident that the oxidation of this compound proceeds beyond the phosphoribonic acid, and even beyond the ketoribonic acid, if this is indeed formed.

Fig. 8 shows the action on various substrates. With this preparation, unlike the system consisting of Lebedew fluid with phenazine methochloride as carrier (Table VI), xylosephosphate and arabinosephosphate are also oxidized, but at less than half the rate of the ribose ester, which in this experiment was also the synthetic compound. It is interesting to note that this is oxidized almost as rapidly as the two hexose phosphoric esters. The action on the arabinose and xylose compounds which did not occur with phenazine methochloride but occurs with the natural carrier may be compared with the different behaviours of Neuberg and Robison esters with phenazine methochloride and flavin enzyme already described [Dickens & McIlwain, 1938].

6. *CO_2 formation.* Fig. 9 shows that CO_2 formation and O_2 consumption are nearly equimolecular.

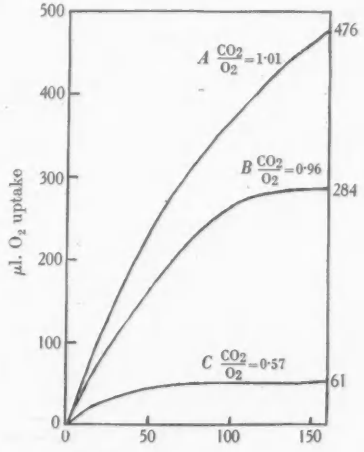

Fig. 9. CO_2 formation and O_2 uptake with dried dialysed Lebedew fluid +0·2 mg. coenzyme. *A*: 0·3 *M*/10 ribosephosph. *B*: 0·1 *M*/10 ribosephosph. *C*: no added substrate.

For this exp. R.Q. vessels [Dickens & Šimer, 1930] were used with enzyme, coenzyme and substrate in the inner part, $Ba(OH)_2$ as CO_2 absorbent, and HCN in the side bulb. The readings were extrapolated for the 1st 10 min. of temperature equalization. During this exp. inorganic P increased by about 0·4–0·5 of the equivalent of the O_2 uptake.

7. *Oxidation by laked blood cells.* Each vessel contained the cells from 2 ml. horse blood, washed three times with 0·9 % NaCl, and diluted to 2 ml. with water. 0·3 ml. *M*/10 substrate was added to each, and 0·1 mg. methylene blue dissolved in 0·2 ml. water was tipped in from the side bulb when equilibrium was reached. 0·2 ml. KOH in inner cup. Fig. 10 shows the O_2 uptakes corrected for the O_2 evolution in the blank [cf. Warburg & Christian, 1931]. Of the three pentose phosphoric acids, the ribose compound is the only one to be attacked. Curves for hexosemonophosphate and phosphohexonate are included for comparison.

8. *Oxidation in tissue slices.* In intact cells diffusion problems probably prevent the entry of phosphorylated products. On the other hand, pentoses are not apparently readily phosphorylated within the cell, though our evidence shows

that they may arise from phosphorylated hexoses which the cell is able easily to manufacture. Consequently, in intact cells oxidation of added pentoses is hardly to be expected, and in fact did not occur (Fig. 11).

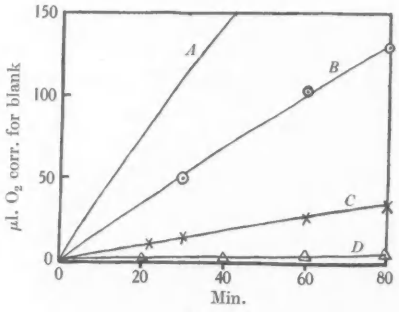

Fig. 10.

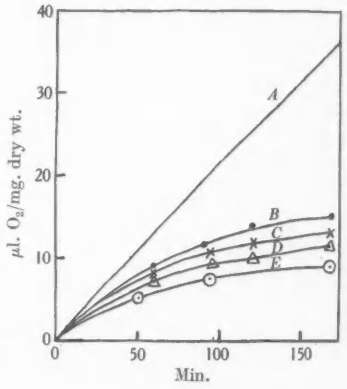

Fig. 11.

Fig. 10. Oxidation by laked horse blood. *A*: hexosemonophosph. *B*: phosphohexonic. *C*: ribose-phosph. *D*: arabinosephosph. *and* xylosephosph.

Fig. 11. Oxidation in brain slices of rat. *M*/90 substrates in phosphate-Ringer solution. O_2. 37·5°. *A*: glucose. *B*: *d*-ribose. *C*: *l*-arabinose. *D*: *d*-arabinose and without added substrate. E: *i*-erythritol.

Although not entirely relevant here, it may be mentioned that kidney slices can readily oxidize 2-keto-*d*-gluconic acid, as the following exp. shows.

Rat kidney slices. Phosphate-Ringer soln. O_2. 37·5°. 0·2 ml. KOH in inner cup.

	Q_{O_2}		
	1st	2nd	3rd hr.
Control	−15·1	−10·9	− 9·6
Na 2-ketogluconate *M*/50	−19·0	−16·7	−15·6

Discussion

It is proposed to discuss these results in detail when a sufficient quantity of the oxidation products has been prepared for their identification. At present the results, particularly the fact that 5-C and 4-C compounds have been identified among the products, provide support for the scheme of progressive oxidation and decarboxylation previously advanced [Dickens, 1936].

The fact that *d*-ribose-5-phosphoric acid, and not the expected *d*-arabinose-5-phosphoric acid, should prove to be the physiologically oxidizable (and fermentable [Dickens, 1938]) substrate introduces a new factor into the problem. Evidently if *d*-ribose is to arise from *d*-glucose by removal of the terminal group, an epimerization must occur. It is recognized that inversion may occur in an asymmetrically disposed C atom with an attached H atom and an adjacent =CO group. A type of Walden inversion could theoretically occur either as a result of decarboxylation or by the introduction and subsequent removal of a second phosphoric acid group. These possibilities will be discussed when more direct evidence of this inversion has been sought.

SUMMARY

I. *Oxidation of phosphohexonate*

An enzyme has been prepared from Lebedew maceration fluid by precipitation at *p*H 4·6, washing with acid and drying. It has been used to study the oxidation of phosphohexonic acid. There is an optimum activity between *p*H 6·3–7·5. The action is inhibited by phosphate like the hexosemonophosphate system, but less powerfully. With pure coenzyme II oxidation of phosphohexonic acid proceeds until $\frac{1}{2}O_2$ per mol. is consumed. At this stage the product, prepared as a Ba salt, appears to consist of a mixture of phosphoketohexonate and phosphopentonic acid, as judged by its analysis and mode of preparation.

When less pure coenzyme is used the reaction proceeds further, the end-products and end-point being altered. The O_2 uptake and CO_2 output are now each about 1 mol. per mol. substrate, an amount different from that seen in experiments of Warburg & Christian [1937] with a different preparation of enzyme. Among the end-products an analytically pure compound corresponding to a phosphorylated 4-C monocarboxylic dihydroxy-acid—possibly phospho-erythronic acid—was isolated. Two other fractions gave a definite reaction for pentose by Bial's test.

II. *Oxidation of pentose phosphoric acids*

Lebedew maceration fluid from dried brewers' yeast oxidizes *d*-ribose-5-phosphoric acid vigorously, and to a less extent *d*-arabinose- and xylose-5-phosphoric acids. When phenazine methochloride is the carrier the latter two substrates are not appreciably oxidized. The activity is destroyed by boiling. The acetate precipitate from Lebedew fluid is feeble towards pentosephosphates, at least with phenazine methochloride as carrier. An active dialysed preparation was made from Lebedew fluid; it can be kept as dry powder and is inactive without the addition of coenzyme II. With coenzyme II oxidation is vigorous with ribosephosphate, only about half as fast with the other pentosephosphates. CO_2 evolution and O_2 absorption are about equal to 1 mol. of each per mol. pentosephosphate. Laked horse blood oxidizes ribosephosphate slightly in presence of methylene blue; the other two pentose esters are not attacked.

Intact cells being impermeable to phosphoric sugar esters do not oxidize them, nor are free pentoses oxidized by brain slices. Kidney slices readily oxidize 2-keto-*d*-gluconic acid.

I wish to express my indebtedness to Dr P. A. Levene for the synthetic pentose phosphoric acids, to Prof. O. Warburg for a gift of pure coenzyme II, and to Prof. R. Robison for an analysed specimen of phosphohexonic acid prepared from Robison ester. Supplies of yeast were kindly given by Newcastle Breweries and Messrs Tennent.

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CCXII. YEAST FERMENTATION OF PENTOSE PHOSPHORIC ACIDS

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(*Received 23 July 1938*)

In all references to pentoses in textbooks on sugars, the fact that they are not fermented by yeast [Salkowski, 1900] is prominent. In the following it is shown that while the pentoses are not fermented appreciably either by yeast cells or by Lebedew extract, phosphorylated pentoses are fermented, and that in the case of the *d*-ribose-5-phosphoric ester, the fermentation by yeast extract is about as rapid as that of glucose or hexosemonophosphate. The fermentation of *d*-ribose-5-phosphoric acid requires the presence of cozymase, yeast enzyme and inorganic phosphate, and probably of metal salts (Mg and Mn) also. (Adenylic acid was present in the cozymase used.) The products of fermentation are per mol. ribosephosphate, 1 mol. CO_2, 1 mol. alcohol and 1 mol. inorganic phosphate. In addition an unknown, HIO_4-reducing substance appears, which may perhaps be glycol.

The natural pentose phosphoric acid of animal and yeast cells—*d*-ribose-5-phosphoric acid—is fermented far more rapidly than either *d*-arabinose-5-phosphoric acid or the corresponding xylose compound. This makes it possible that *d*-ribose-5-phosphoric acid may play an important part as an intermediate both in aerobic fermentation and in sugar oxidation [Dickens, 1938]. These facts may eventually prove of significance in connexion with the mechanism of the Pasteur reaction and in carbohydrate metabolism generally. The part played by pentose phosphoric acids in metabolism is being further studied.

Methods

Alcohol estimation. The method of Friedemann & Klass [1936] was used and proved satisfactory provided that all the precautions described were rigidly observed. Erratic results are obtained unless all glassware is cleaned with chromic-sulphuric acid immediately before use. The distillation apparatus was modified to suit existing equipment, and with the glass condenser used the recovery was as follows: μl. alc. taken (1 m*M*.$=22400\,\mu$l.); 224, 224, 45, 45; recovered; 90, 91, 93·5, 88% mean recovery 90·5%. Redistillation from $Ca(OH)_2$-$HgSO_4$ was always used after the distillation from $HgSO_4$, to ensure removal of interfering substances. Pentose phosphoric esters did not give any volatile titratable material. It is essential to have a large excess of $KMnO_4$ during oxidation.

Reducing substances. The Cu reduction method of Somogyi [1937] was adapted for the separate estimation of pentose and pentose phosphoric acid. The reducing values of *d*-ribose, *d*-ribose-5-phosphoric acid and glucose found for our preparations were respectively 58%, 57% and 100% for equimolecular quantities (ribose-phosph. calc. on P content of soln. of Na salt). Neutral Pb acetate was found to remove ribosephosphate practically quantitatively from its solution and the loss of free ribose under the same conditions was negligible.

Method. The proteins are removed with tungstic acid as usual. The perfectly clear centrifugate is pipetted into a dry centrifuge tube, an aliquot being used for total reducing substance. The ribosephosphate is precipitated by neutral Pb acetate, allowing to stand for ½ hr. Centrifuging removes the lead salt and the clear or faintly opalescent supernatant fluid is pipetted into a dry centrifuge tube, excess $M/2$ Na_2HPO_4 added, Pb phosphate removed by centrifuging and a measured amount of the perfectly clear supernatant fluid used for the estimation of reducing substance not precipitable by neutral Pb acetate. 0·03 ml. $M/10$ solution of ribosephosphate per ml. tungstate filtrate was the concentration usually employed for the Pb precipitation.

HIO_4 titration. This was used for detection and estimation of substances containing two or more adjacent free OH groups (micro-method modified from Fleury & Fatome [1935]). It was found that 1 ml. $M/50$ soln. of each of the following required the approximate amounts of $N/50$ I_2 stated: glycerol, 4·0; glucose, 8·8; ribose, 6·4; hexosemonophosphate, 7·1; ribosemonophosphate, 5·3; glycol, 2·0 ml. The method is applicable to the Na_2HPO_4 supernatant fluid mentioned above, provided that it is acidified to litmus before adding the HIO_4. Quantities below 1 ml. $M/100$ can be estimated with accuracy, but the method is only applicable if sugars and interfering substances are known to be absent.

Materials

Pentose-5-phosphoric acids. The natural *d*-ribose-5-phosphoric acid and synthetic *d*-arabinose- and xylose-5-phosphoric acids (Levene) used have already been described [Dickens, 1938], together with the prep. of the $M/10$ soln. of the Na salts. *d-Ribose* was made from guanosine (B.D.H.) according to Levene & Clark [1921]. It crystallized readily in the desiccator.

Cozymase was a purified prep. made in this laboratory by Dr Weil-Malherbe.

Muscle adenylic acid was obtained from Fraenkel and Landau. It appeared to contain cozymase, and as the cozymase contained adenylic acid, adenylic acid was not added in more than a few exp.

Mg-Mn soln. 0·025 M $MgSO_4$ containing 0·005 M $MnSO_4$.

Ca hexosediphosphate B.D.H. prep. *Mg hexosediphosphate* kindly presented by Prof. Schoeller, Schering-Kahlbaum. $M/50$ soln. was used, the Ca salt being dissolved by partial decomp. by Na_2SO_4.

Glycollaldehyde was prepared from dihydroxymaleic acid by distillation after heating with pyridine [Fischer & Taube, 1927]: it crystallized readily after distillation.

Enzyme preparations. Lebedew fluid was prepared from Löwenbräu, Munich, bottom yeast. The dialysed, dried preparation already described [Dickens, 1938] was also made from this yeast.

Fermentation of pentose and pentosephosphoric acids by Lebedew fluid and by yeast cells

Yeast cells. D.C.L. Company's yeast was washed three times at the centrifuge with dist. water and a 2% suspension in $M/25$ KH_2PO_4 prepared. 1 ml. per vessel was transferred to Warburg manometers and the substrates were added to make the conc. $M/15$. Fermentation was measured in N_2 at 37·5° (Fig. 1). Ribose was not at all fermented, ribosephosph. was slightly attacked, but the action was very small compared with the very vigorous fermentation of glucose and may have been due to a trace of impurity, but in such cases there is usually a sharp fall of reaction rate, which was not so here.

Lebedew fluid. Fig. 2 shows that contrary to what happens with the intact cells, fermentation with ribose phosph. is very vigorous in Lebedew extract, and ribose is also attacked, though relatively very much more slowly.

Products and end-point of fermentation of ribosephosphate by Lebedew fluid. Fig. 3 shows the course of the reaction. The end-point of fermentation of 9 ml.

Lebedew fluid with 2·4 ml. 0·76 *M*/10 ribosephosph. was 7840 μl. CO_2; blank (9 ml. + 2·4 ml. water) 735 μl. Diff. 7105 μl. CO_2. Ribose P taken = 8170 μl. (22400 μl. = 1 ml. *M* soln.). Ratio: mol. CO_2/mol. ribose P = 0·86.

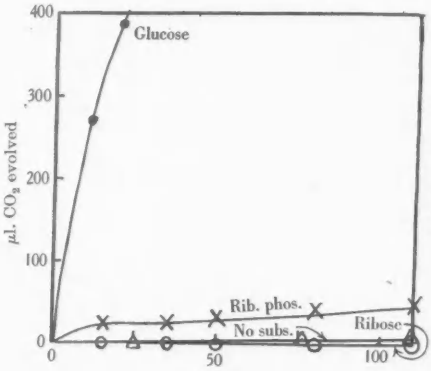

Fig. 1.

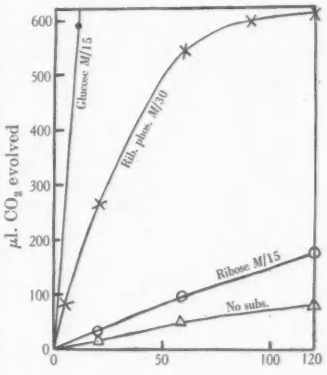

Fig. 2.

Fig. 1. Yeast cells. Fermentation of glucose, ribose and ribosephosphate. Conc. *M*/15.

Fig. 2. Fermentation with Lebedew macerate (1 ml.). Conc. of substrates in mixture are shown.

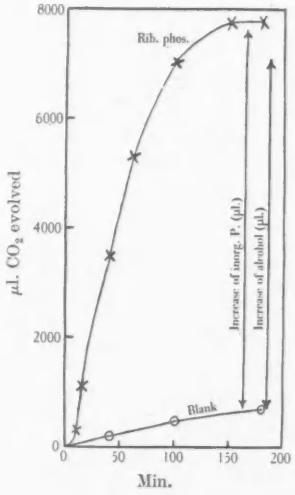

Fig. 3.

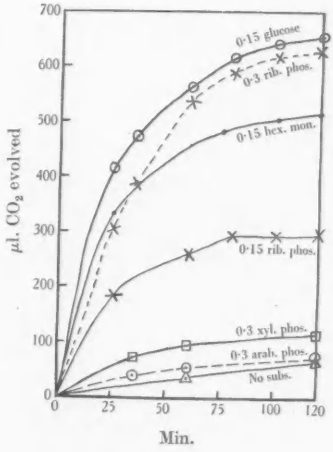

Fig. 4.

Fig. 3. Fermentation of ribosephosphate by Lebedew fluid: end-point, alcohol and P estimations.

Fig. 4. Fermentation of various substrates by Lebedew fluid (1 ml.): ml. *M*/10 substrates added as shown.

Increase of inorganic P in trichloroacetic acid filtrate = 6950 μl. = 98 % of CO_2 evolved and = 85 % of ester P present in the ribosephosph. Alcohol increase, 6300 μl. = 90 % of CO_2 formed (1 m.Mol. H_3PO_3 or C_2H_5OH = 22,400 μl.)

This exp. shows that ribosephosph. is decomposed to the extent of 86 % by Lebedew fluid; that CO_2 equivalent to 85 % of 1 mol. CO_2 per mol. total ribosephosph. is liberated; that the alcohol formed, assumed to be ethyl alcohol, is 77 % of the total ribose ester taken, and that inorganic phosphate is split off equivalent to the CO_2 formed.

Fermentation of other pentose phosphoric acids by Lebedew fluid

Fig. 4 shows the result of incubating various substrates with Lebedew fluid. Arabinosephosph. and xylosephosph. are only slightly fermented. With ribosephosph. we have seen that only 1 mol. CO_2 is liberated per mol. substrate, hence to compare the rate of reaction with those of hexosemonophosphate and glucose, which give 2 mol. CO_2 per mol., half quantities of these substrates were used (Fig. 4). When this is done, the rate of fermentation of ribosephosph. is close to that of glucose, and about equal to that of hexosemonophosphate (Robison ester).

In view of the fact that the pentoses themselves are practically non-fermentable, this observation opens a new field for investigation.

The alcohol estimations in the exp. of Fig. 4 were as follows:

Table I. *Alcohol estimations and CO_2 liberation*

Substrate	Water	0·3 ml. 0·05 *M* glucose	0·15 ml. 0·091 *M* hex. mono-phosph.	0·3 ml. 0·076 *M* ribose phosph.	0·3 ml. 0·08 *M* arab. phosph.	0·3 ml. 0·08 *M* xyl. phosph.	0·15 ml. 0·076 *M* rib. phosph.
μl. substrate	0	336	303	511	538	538	256
μl. CO_2 evolved	64	656	514	625	75	115	298
Increase CO_2 over blank	0	592	450	561	11	51	234
Calc. CO_2 for mols.	—	2	2	1	1	1	1
,, μl.	—	672	606	511	538	538	256
$\left(\frac{\text{Found}}{\text{Calc.}}\right)$ CO_2 %	—	88	74	110	2	9·5	91
μl. alcohol (increase over blank)	—	732	—	582	—	—	—

With Lebedew fluid, the alcohol increase, being small in proportion to the relatively high alcohol content of the macerate, can only be measured approximately. The CO_2 retention of Lebedew fluid, despite its acid reaction, is also appreciable. These objections apply less to the enzyme experiments.

Fermentation of ribosephosphate by dried enzyme

Each Warburg vessel contained 80 mg. dried dialysed Lebedew extract dissolved in 1 ml. water and centrifuged. 0·1 ml. Mg-Mn soln., *M*/2 KH_2PO_4, *M*/50 hexose-diphosph., adenylic acid (1 mg./ml.), and cozymase (2 mg./ml.), and 0·2 ml. acetaldehyde (25 mg./ml.) and glycollaldehyde (25 mg./ml.) were added as required. In all exp. unless otherwise stated 0·3 ml. *M*/10 ribosephosphate and 0·3 or 0·15 ml. *M*/10 hexosemonophosphate were the substrates used. N_2. 37·5°.

The results are shown in Figs. 5–7.

Induction period. Fig. 5 shows that fermentation with the dried enzyme sets in only after the addition of an activator (in one exp. a spontaneous onset of fermentation after 30 min. was observed; this may have been due to accidental

contamination with some activating substance). In the exp. of Fig. 5 the actions of acetaldehyde with ribosephosphate and hexosemonophosphate are compared.

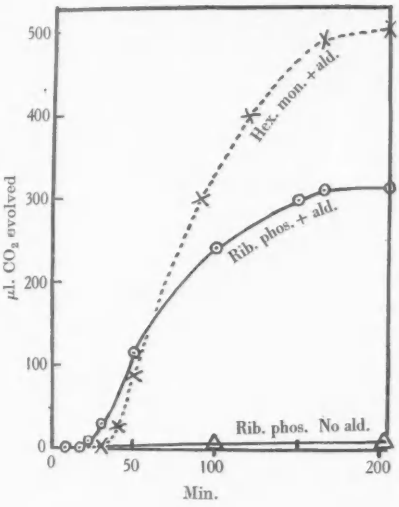

Fig. 5.

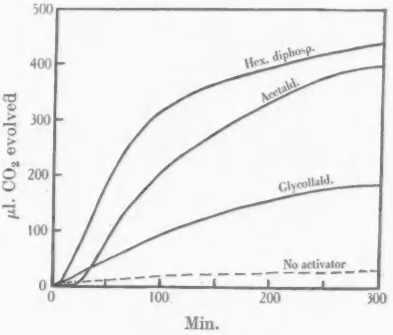

Fig. 6.

Fig. 5. Dried enzyme: activation of fermentation of ribosephosph. by acetaldehyde.

Fig. 6. Dried enzyme: comparison of activators (acetald. $M/20$, glycollaldehyde $M/30$, hexosediphosphate $M/900$).

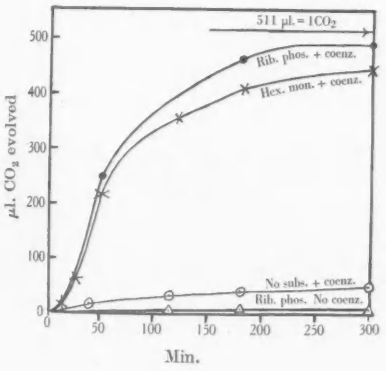

Fig. 7.

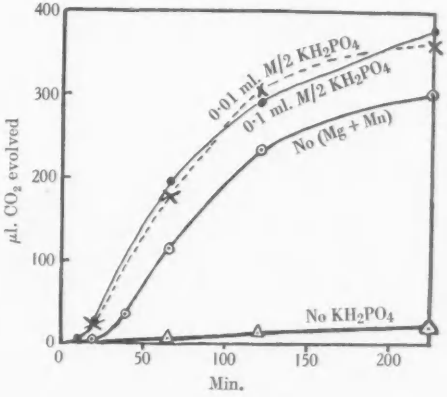

Fig. 8.

Fig. 7. Dried enzyme: need for coenzyme I. 0·3 ml. ribosephosph., 0·15 ml. hexosemonophosph.

Fig. 8. Dried enzyme: need for inorg. P; effect of addition of Mg and Mn.

Fig. 6 gives a comparison of the activation by acetaldehyde with that of hexosediphosphate and glycollaldehyde. Glycollaldehyde was tested because it has often been suggested as a breakdown product of pentoses and was described by Neuberg & Sandberg [1921] as an exceptionally vigorous activator in hexose fermentation. It was less efficient than the other two substances, of which hexosediphosphate was the better. (0·1 ml. $M/50$ hex. diphosph. = 45 μl. per vessel.) The action resembles that well known in hexose fermentation. This amount of hexosediphosphate was added in all the following experiments.

Coenzyme. Fig. 7 shows that cozymase is necessary for fermentation to proceed, and also shows the end-point of the fermentation clearly. Many similar results were obtained. The cozymase used was that tested previously [Dickens, 1938] and shown to be free from coenzyme II; it probably contained adenylic acid.

Phosphate and mineral salts. Fig. 8 shows that inorganic phosphates are a necessary component for fermentation to occur. (Dialysed enzyme free from inorg. P; other reagents also, except ribosephosph. which contained 0·06 mg. (40 μl.) inorg. P in the 0·3 ml. used.) Addition of 112 μl. inorg. P produced full activation, smaller quantities were not tested. By analogy with hexose fermentation this suggests the primary formation of a pentosediphosphate; evidence for which is being sought.

It is also clear from Fig. 8 that the reaction is influenced by the presence of Mg^{++} and Mn^{++}. (0·1 ml. Mn-Mg soln. added contained 55 μl. Mg and 11 μl. Mn.) Special precautions would be necessary to obtain an enzyme prep. free from traces of these metals.

The fermentation of pentose phosphoric acid was in all these respects like that of hexosemonophosphate, which has been so much studied.

Alcohol estimations

Table II. *Alcohol estimations with dried enzyme*

Substrate	Coenzyme	Activator	Time of exp. (min.)	Alcohol μl.	CO_2 evolved μl.
Ribosephosph.	None	None	150	11	0
			240	0	0
,,	+	None	240	69	32
,,	+	Acetald.	240	475	397
,,	None	Hexosediphosph.	240	26	11
,,	+	,,	240	369	439
Water	+	,,	240	38·5	66·5
Hexosemonophosph.	None	,,	310	5·5	1·5
,,	+	,,	310	624	682

The results show that the CO_2 output is on the whole in reasonably good agreement with the alcohol formation, on the basis of the assumption that 1 ml. pentose ester gives 1 mol. CO_2 and 1 mol. alcohol. A great many experiments and long practice with the micro-estimation would be necessary to prove the exact degree of agreement.

In view of the fact that in the one exp. with acetaldehyde 1100 μl. of this substance were added, the degree of its removal may be regarded as fairly satisfactory, and makes it very improbable that volatile aldehydes formed in the fermentation could interfere seriously with the alcohol estimation.

The exp. of Table III shows that nearly all the ester P added is split off during the incubation, whether fermentation occurs or not (with or without cozymase).

Table III. *Phosphate balance sheet*

Vessel no. ...	1	2	3	4	5
Contents of vessel:					
A. Enzyme + KH_2PO_4 + Mg + Mn	+	+	+	+	+
B. Hexosediphosph. 0·1 ml. *M*/50	+	+	+	+	+
C. Cozymase, 0·2 mg.	−	+	+	−	+
Substrate:					
D. Ribosemonophosph. 0·3 ml.	+	+	−	−	−
E. Hexosemonophosph. 0·15 ml.	−	−	−	+	+
310 min. incubation. N_2. 37·5°					
Inorganic P mg. added in component:					
A	1·37	1·37	1·37	1·37	1·37
B	0·00	0·00	0·00	0·00	0·00
C	0·00	0·00	0·00	0·00	0·00
D	0·06	0·06	0·00	0·00	0·00
E	0·00	0·00	0·00	0·03	0·03
Total at start	1·43	1·43	1·37	1·40	1·40
Found at end	2·16	2·21	1·53	1·81	1·87
Increase inorg. P	**0·73**	**0·78**	**0·16**	**0·41**	**0·47**
Organic P (acid hydrolysable, not precip. trichloroacetic acid) mg. added in component:					
A	0·00	0·00	0·00	0·00	0·00
B	0·13	0·13	0·13	0·13	0·13
C	0·00	0·01	0·01	0·00	0·01
D	0·71	0·71	0·00	0·00	0·00
E	0·00	0·00	0·00	0·38	0·38
Total at start	**0·84**	**0·85**	**0·14**	**0·51**	**0·52**
$\frac{\text{Increase inorganic P} \times 100}{\text{Total organic P added}}$ %	87	92	110	80	90

Loss of reducing substance on fermentation

Table IV shows the balance sheet of an experiment in which the increase of inorganic P is correlated with the appearance of a non-precipitable[1] reducing

Table IV. *Incubation of ribosephosphate with and without coenzyme*

	No coenzyme		+Coenzyme	
Vessel no.	1 Preformed	2 Final	3 Preformed	4 Final
Incubation time, min.	0	330	0	330
Inorg. P, μl. (31 mg. = 22,400 μl.)	1530	2370	1530	2400
Ribosephosph. ester-P added, μl.	1020	1020	1020	1020
	Increase on		Increase on	
Increase inorg. P, μl.	no. 1	840	no. 3	870
CO_2 evolved, μl.	—	11·5	—	845
Total Cu-reducing subs. per vessel, calc. as ribose (or ribosephosph.), μl.	1031	1386	1031	342
Change during exp., μl.	+337		−689	
Non-precipitable Cu-reducing subs., μl.	144	922	144	160
Change during exp., μl.	+778		+16	
Orcin-$FeCl_3$ test on Pb acetate supernatant liquid	Trace	+++	Trace	Negative
Periodate titration on Pb acetate supernatant liquid: I_2 equiv. of HIO_4 reduced; ml. *N*/100 I_2	8·8	30·2	8·4	18·7
Increase during exp.	21·4		10·3	
Calc. I_2 equiv. of reducing substance liberated (assumed to be ribose)*	22·6		0·5	

* Taking 1 ml. *M*/100 ribose (= 224 μl.) = 6·5 ml. *N*/100 I_2.

[1] Using the term to mean not precipitated by neutral Pb acetate.

substance, presumably ribose, in the absence of coenzyme, and with the virtually complete disappearance of reducing substance in excess of that of the blank, when ribosephosphate is incubated in the presence of cozymase.

The results of Table IV show that pentose, as indicated by the orcin-$FeCl_3$ test, completely disappears from the vessel containing ribosephosphate and coenzyme after fermentation is complete (ribosephosph. gives the test as strongly as free ribose). There is an increase in the HIO_4 titration on the Pb non-precipitable fraction which does not correspond with the small change of reducing value of the same fraction. It suggests the formation of a new product during fermentation, and from the fact that this substance reduces HIO_4 it probably contains two adjacent OH groups.

Nature of the HIO_4-reducing substance. It has been shown above that the fermentation of 1 mol. *d*-ribose-5-phosphoric acid results in the formation of 1 mol. CO_2, 1 mol. alcohol and 1 mol. H_3PO_4. The residue of the 5-C chain remains to be accounted for:

$$\underset{\text{d-ribose-5-phosp.}}{C_5H_9O_5.PO_3H_2} + H_2O = \underset{\text{ethyl alc.}}{C_2H_5.OH} + H_3PO_4 + CO_2 + \underset{\text{Unknown 2-C product}}{C_2H_4O_2}$$

The most obvious choice would be glycollaldehyde, which arises during the action of Na_2CO_3 on pentoses [Fischler & Boettner, 1928] together with methylglyoxal, the latter possibly from intermediate formation of triose. Glycollaldehyde has also been suggested as an intermediate in the fermentation of pentose by the pentose-fermenting bacteria by Peterson & coworkers and by van der Lek [lit. quoted Kluyver, 1935]. Titration of the $NaHSO_3$-combining substances and distillation of volatile acids revealed no significant increase, however; hence neither glycollaldehyde nor acetic acid appears as a main product. It should be possible by fixation experiments to obtain evidence of the intermediate formation of glycollaldehyde, if this occurs. This is now being tested.

Neuberg & Schwenk [1916] found that glycollaldehyde was reduced by vigorously fermenting yeast to form glycol. The following exp. however suggests that glycollaldehyde, if formed, would probably undergo dismutation under our conditions.

200 mg. dialysed dried Lebedew prep. were dissolved in 2 ml. water + 1 ml. 1·3% $NaHCO_3$. 0·4 mg. cozymase was added and the soln. (1 ml.) placed in the main part of three Warburg vessels. $N_2 + CO_2$, 37·5°. The side bulbs contained 5 mg. glycollaldehyde, 5 mg. acetaldehyde and water respectively. A steady positive pressure developed only in the vessel with glycollaldehyde. It amounted to the equiv. of 100 μl. CO_2 per hr.: water control 12 μl. CO_2 per hr.

The nature of the products formed is being investigated, but it is probable that dismutation to glycollic acid and glycol had occurred, with displacement of CO_2 from the bicarbonate.

This result shows that the formation of glycol ($\frac{1}{2}$ mol.) and glycollic acid ($\frac{1}{2}$ mol.) from glycollaldehyde (1 mol.) could occur with our enzyme prep. As yet however tests for glycollic acid [Denigès, 1909] have not been successful. On the other hand, there is an increase in the HIO_4-titratable material after fermentation, which might indicate the presence of glycol. The substance is non-Cu-reducing and is not precipitable by neutral Pb acetate and the facts are consistent with its being glycol, except that its reducing value towards HIO_4 exceeds that calc. for $\frac{1}{2}$ mol. glycol. per mol. pentose. The nature of this substance will be further investigated.

Discussion of these results will be postponed until the fate of the 2-C moiety is cleared up. In the meantime, however, attention may be drawn to the requirement for inorganic P if fermentation is to proceed, suggesting the need for formation of a diphosphate, analogous to that found in hexose fermentation. Recently Dische [1938] has made the interesting observation that *d*-ribose combined in adenosine is phosphorylated by inorganic phosphate in blood haemolysate, and that the phosphorylated ribose is afterwards broken down to a number of products, including probably triosephosphate and glycollaldehyde or glycollaldehyde phosphate. Phosphorylation and fermentative cleavage of ribosephosphates may thus occur in animal cells also.

Summary

d-Ribose-5-phosphoric acid is readily fermented by Lebedew macerate of dried beer yeast with the formation of 1 mol. alcohol, 1 mol. CO_2, 1 mol. free phosphoric acid and an as yet unindentified product.

Other pentose-5-phosphoric acids are very little fermented (*d*-arabinose and xylose esters were used). The rate of fermentation of ribosephosph. is similar to that of glucose or hexosemonophosphate (Robison ester), when allowance is made for the fact that these substances both give 2 mol. CO_2 and alcohol, instead of only the one given by ribosephosph. As would be expected, intact yeast cells hardly ferment ribose or its phosphoric ester.

An enzyme preparation free from coenzyme and phosphate required the addition of cozymase and phosphoric acid before fermentation set in. An activator (hexosediphosphate, acetaldehyde, glycollaldehyde) is needed to remove the delay in onset of fermentation, as with hexosephosphate fermentation in similar preparations.

The products of the reaction with the dried enzyme, cozymase, inorg. P, Mg and Mn salts, are the same as with Lebedew extract as far as the alcohol, CO_2 and inorg. P are concerned.

The experiments are being continued to determine the nature of the remaining product. It does not reduce alkaline Cu reagent, is not precipitated by neutral Pb acetate and appears to contain two or more adjacent unsubstituted hydroxyl groups.

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CCXIII. THE LACTIC DEHYDROGENASE OF LACTIC ACID BACTERIA

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The occurrence of lactic dehydrogenase in lactic acid bacteria has not yet been demonstrated, although many authors have already proved the presence of the dehydrogenase in animal tissues and various micro-organisms.

The specificity of the form of lactic acid dehydrogenated by the enzyme has been pointed out by several authors. Meyerhof & Lohmann [1926] observed that *d*-lactic acid was more rapidly oxidized than the *l*-acid by yeast and animal tissues including muscle, liver, kidney and brain. The same conclusion was obtained by Banga *et al.* [1932] with muscle. Green & Brosteaux [1936] observed that the enzyme of heart muscle showed complete optical specificity in oxidizing only *d*-lactic acid, while *Bact. coli* oxidized the *l*-component more rapidly and the yeast preparation preferentially attacked the *d*-component.

In the present paper, the presence of lactic dehydrogenase was verified in all the kinds of lactic acid bacteria under investigation, and the enzymes of these bacteria were found to show complete optical specificities.

Methods

The various kinds of lactic acid bacteria were cultivated on 600 ml. koji extract containing 24–30 g. sugar and 30 g. $CaCO_3$ at 30° for 5 days. The bacterial cells were collected on the centrifuge after being separated from calcium carbonate by decantation. The cells were then suspended in 100 ml. water and strongly aerated. After being washed several times with water, the cells were finally suspended in 50 ml. of water and again aerated.

For the detection of dehydrogenase, the reduction time of methylene blue at 40° was measured anaerobically (less than 20 mm.) in a Thunberg tube into which 0·2 ml. of the bacterial suspension (in the case of *Lactob. sake* 0·4 ml. was used) was put with 0·2 ml. 5 % Ca lactate solution and 1·8 ml. methylene blue solution prepared by mixing 8 ml. 0·05 % methylene blue and 6 ml. of 0·2 *M* phosphate buffer adjusted to *p*H 7·2.

Lactobacillus sake (d-*acid-former*)

When the experiments were carried out with *Lactob. sake* No. 84, it will be seen in Table I that reduction of methylene blue was observed in the presence

Table I. *Specificity of lactic dehydrogenase*

	Reduction time of methylene blue (min.)		
Donator	*d*-Acid-former	*l*-Acid-former	*dl*-Acid-former
d-Lactic acid	21	>180	25
l-Lactic acid	>180	11	13
dl-Lactic acid	20	17	15
Water (control)	>180	>180	>180

of *d*- and *dl*-lactic acids. Therefore the bacterial cells were found to show complete optical specificity in oxidizing only *d*-lactic acid, as was already pointed out by Green & Brosteaux [1936] with heart muscle extract.

Leuconostoc mesenteroides (l-*acid-former*)

In contrast with the case of *Lactob. sake* mentioned above, only the *l*-component of lactic acid was available to *Leuconostoc mesenteroides* var. *sake* as donator (see Table I).

It is of interest that the optical specificities of the lactic acids dehydrogenated by these bacterial cells coincide with those of the acid produced by the bacteria themselves. Lactic dehydrogenase can therefore be classified into *d*- and *l*-enzymes.

Lactobacillus plantarum (dl-*acid-former*)

It will be seen in Table I that *Lactob. plantarum* sp. dehydrogenated all the optical components of lactic acid, although the *l*-component was attacked a little more rapidly.

Thus all the kinds of lactic acid bacteria mentioned above effected dehydrogenation of lactic acid in the presence of methylene blue. No oxidation of lactic acid was ever observed in the absence of methylene blue.

In order to dehydrogenate all the optical components of lactic acid, the presence of both *d*- and *l*-lactic dehydrogenases would not be absolutely necessary, since all forms of the acid could be attacked by any one of the dehydrogenases in presence of racemiase, with which the racemic form of lactic acid was always produced as has already been pointed out by us [1937].

It was found by us [1938] that racemiase in *Lactob. plantarum* was easily inactivated by treatment with acetone, while lactic dehydrogenase would not be inactivated by acetone, as was pointed out by Harden & Macfarlane [1931] with yeast. Therefore the experiments shown in Table II were carried out with acetone-dried bacteria in order to investigate the effect of racemiase upon the dehydrogenation of lactic acid.

Table II. *Effect of racemiase*

Lactob. plantarum *dl*-Acid-former	Reduction time of methylene blue (min.)		
	d-Lactic acid	*l*-Lactic acid	Water
Resting cells	38	18	>180
Acetone-dried cells	>180	20	>180

It will be seen in Table II that acetone-dried cells never attacked *d*-lactic acid, while *l*-acid was as easily dehydrogenated by it as by the resting cells.

The reason why *Lactob. plantarum* attacked all the forms of lactic acid was thus attributable not to the presence of both *d*- and *l*-lactic dehydrogenases, but to that of *l*-lactic dehydrogenase co-operating with racemiase.

The product of oxidation

The identification of pyruvic acid produced by oxidation of lactic acid with lactic dehydrogenase is not easy to accomplish, since decomposition of the pyruvic acid by carboxylase usually occurs or α-ketonoxidase is present in the dehydrogenase preparations.

In the present experiments, it was found that pyruvic acid could be directly isolated when lactic acid bacteria were used for the dehydrogenase.

(*a*) *Lactobacillus plantarum*. The experiments were carried out with seven large (100 ml.) Thunberg tubes. Each tube, containing 10 ml. 0·2 *M* Na *dl*-lactate, 10 ml. 0·2 *M* phosphate buffer (*p*H 7·2), 20 ml. 0·5 % methylene blue and 20 ml. bacterial suspension, was evacuated and kept at 40°. When the methylene blue was almost reduced, another 20 ml. of the methylene blue solution were added and the experiments were continued in exactly the same manner until decoloration of the dye was complete, which was attained in about 2 hr. throughout the whole of the experiments.

The decolorized solutions were collected, the dye adsorbed first with kaolin and then with active C. The clear solution thus obtained was acidified with H_2SO_4 and the pyruvic acid extracted by ether.

0·4 g. of yellowish acicular crystals, M.P. 184°, was obtained by the addition of phenylhydrazine hydrochloride solution to the aqueous solution of the acid after removal of the ether. After recrystallization the M.P. was found to be 192° which was identical with that of the phenylhydrazone of pyruvic acid. (Found: C, 60·49; H, 5·73 %. $C_9H_{10}O_2N_2$ requires C, 60·62; H, 5·62 %.)

The yield was 51 % of that calculated from the equation:

$$CH_3.CHOH.COOH + Mb = CH_3.CO.COOH + MbH_2$$

(*b*) *Lactobacillus sake*. The experiments were carried out in the same manner as was mentioned above, with two Thunberg tubes in which were placed 10 ml. 0·2 *M* Na *d*-lactate, 10 ml. 0·2 *M* phosphate buffer, 5 ml. 0·5 % methylene blue and 20 ml. of the bacterial suspension.

The solution was analysed after 5 hr., and 0·016 g. of the phenylhydrazone of pyruvic acid was obtained; yield found, 60 %.

(*c*) *Leuconostoc mesenteroides* var. *sake*. The same experiments as were instituted with *Lactob. sake* were carried out with *l*-lactate. The decoloration of the 15 ml. 0·5 % methylene blue in each Thunberg tube was attained within 6 hr. but the tubes were kept for a further 6 hr., in order to test whether pyruvic acid would be decomposed by the bacteria.

The yield of pyruvic acid was observed to be 58 %, since 0·049 g. of the phenylhydrazone was obtained from the two tubes.

No evidence of the production of acetaldehyde or acetic acid, which would be derived from pyruvic acid by carboxylase or α-ketonoxidase, was ever obtained in these experiments.

Thus it was clearly demonstrated that lactic dehydrogenase existed in all the lactic acid bacteria and that carboxylase could not be detected even in *Leuconostoc*, which converts glucose into lactic acid, alcohol and CO_2. This is in disagreement with the suggestion of Peterson *et al.* [1922] that the heterofermentation revealed by such types of bacteria as *Leuconostoc* would be caused by the presence of carboxylase.

Summary

1. The occurrence of lactic dehydrogenase was verified with various kinds of lactic acid bacteria.

2. It was found that a *d*-acid-former (*Lactob. sake*) dehydrogenated only *d*-lactic acid and an *l*-acid-former (*Leuconostoc*) attacked only the *l*-acid. Lactic dehydrogenase is therefore considered to be a mixture of *d*- and *l*-enzymes.

3. The reason why a *dl*-acid-former (*Lactob. plantarum*) attacked all forms of lactic acid was not the presence of both kinds of lactic dehydrogenases, but the co-operation of racemiase with the *l*-enzyme.

4. The occurrence of carboxylase was never detected and pyruvic acid was easily isolated, in a yield of about 60%, as the oxidation product of lactic acid by any of the bacteria used.

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CCXIV. A SURVEY OF ANTHOCYANINS. VI

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In earlier parts of this series [Robinson & Robinson, 1931; 1932; 1933; 1934; Lawrence *et al.* 1938] the nature of the anthocyanins present in the flowers, fruits, permanently pigmented leaves and autumn leaves of many species of plants has been described. To complete the analysis of the distribution of the principal anthocyanin types in the Angiosperms, a series of young leaves has now been examined. In young leaves coloration due to anthocyanins, disappearing with maturity, is a phenomenon which is probably more general than autumnal anthocyanin development, and the (approximately) 200 species, in 110 genera, tested were chosen to cover as wide a range of plants as possible.

As in autumn leaves, cyanidin saccharides predominate, being found in 93% of the genera. In a few cases, other constituents interfered with the colour reactions and distribution, rendering it difficult to identify the glycosidal type definitely. But in those genera which contained cyanidin derivatives whose sugar types were determined, there were found:

31% monoglycosides	30% 3-biosides
50% pentoseglycosides	9% 3:5-dimonosides

A comparison of the figures with those derived from autumn leaves, flowers etc. will be made in a later communication.

Results

Figures refer to the numbers given by Hutchinson [1926] in his classification of the flowering plants, the first indicating the order and the second the family, e.g. 76–264 order Lamiales, family Labiatae. For completeness two plants have been included which were recorded previously, namely *Impatiens biflora* and *Crataegus oxyacantha fl. pl. rosea* [Robinson & Robinson, 1932].

Dicotyledons

76–264.	*Ajuga reptans* var. *atropurpurea*	o	67–238.	*Aster ericoides*	j
	Elsholtzia Stauntonii	b		*Silphium perfoliatum*	d
	Lamium purpureum	d		*Solidago aurea*	g
	Salvia nemorosa	o		*Sonchus oleraceus*	a
	S. officinalis	k	66–233.	*Abelia chinensis*	n
	S. virgata	m		*Lonicera Ferdinandii*	j
76–263.	*Callicarpa koreana*	g		*L. Maackii*	g
	Clerodendron foetidum	a		*L. Periclymenum*	j
	C. trichotomum	a		*L. Periclymenum* var. *belgica*	j
	Petraea volubilis	v		*L. pileata* var. *yunnanensis*	g
75–257.	*Bignonia Unguis-cati*	g		*Symphoricarpus orbiculatus* var. *conglomeratus*	g
75–256.	*Streptocarpus Rexii*	q		*S. occidentalis*	g
75–252.	*Pentstemon procerus*	l		*Viburnum betulifolium*	o
	Veronica Chamaedrys	j		*V. fragrans*	a
74–250.	*Solandra Hartwegii*	c		*V. Opulus* var. *sterile*	h
69–241.	*Ceratostigma plumbaginoides*	y		*V. rugosum*	d
	C. Willmottianum	x		*V. Tinus* var. *hirtum*	h
	Limonium latifolium	A	65–230.	*Apocynum cannabinum*	d
69–240.	*Dodecatheon Meadia*	o	64–229.	*Jasminum officinale*	d
	Steironema ciliatum	w			

64–229.	*Jasminum primulinum*	d
	J. revolutum	e
	Ligustrum Quihoui	h
	L. sinense	d
	Osmanthus Delavayi	f
	Syringa Sweginzowii var. *superba*	h
	S. vulgaris	f
60–216.	*Agapetes buxifolia*	b
	Pentapterygium serpens	a
	Vaccinium caespitosum	a
	V. pennsylvanicum	A
	V. virgatum	a
60–215.	*Erica cinerea*	a
	Macleania insignis	a
	Pieris japonica	a
60–214.	*Clethra arborea*	d
59–213.	*Bupleurum fruticosum*	a
59–212.	*Hedera Helix*	g
57–200.	*Acer cappadocicum* var. *rubrum*	a
	A. Ginnala	d
	A. palmatum var. *septemlobum elegans*	d
57–198.	*Koelreuteria paniculata*	g
55–194.	*Skimmia japonica*	a
54–193.	*Vitis Henryana*	B
51–173.	*Euonymus oxyphyllus*	i
51–171.	*Ilex Aquifolium*	d
50–169.	*Urtica dioica*	h
43–156.	*Populus monilifera*	d
42–151.	*Hamamelis japonica* var. *flavopurpurascens*	z
42–150.	*Stachyurus praecox*	g
41–148.	*Physostegia virginiana*	g
40–145.	*Calycanthus floridus*	d
40–143.	*Amelanchier canadensis*	d
	Aronia melanocarpa	d
	A. arbutifolia	e
	Cotoneaster acutifolia	d
	C. bullata	a
	C. Dielsiana	d
	C. horizontalis	a
	Crataegus congestiflora	a
	C. oxyacantha var. *flore pleno rosea*	d
	Cydonia japonica	d
	C. lagenaria	d
	C lagenaria var. *aurea*	d
	Exochorda racemosa	g
	Malus baccata var. *Jackii*	e
	M. pumila var. *Niedzwetzkyana*	a
	M. Sieboldii	b
	M. yunnanensis var. *Vietchii*	a
	Neillia longiracemosa	d
	N. opulifolia var. *lutea*	b
	Photinia serrulata	e
	Poterium tenuifolium var. *album*	n
	Prunus cerasifera var. *Blirieana*	d
	P. cerasifera var. *Moseri*	d
	P. serrulata var. *forma*	d
	P. serrulata var. *pubescens*	d
	Pyracantha atalantioides	d
	Pyrus purpurea	a
	Raphiolepis indica	a
	Rosa Banksiae	j
	R. filipes	h
	R. multiflora var. *cathayensis*	i
	R. omeiensis var. *praecox*	d
	Rubus thyrsiger	o
	Sorbus hupehensis var. *rosea*	a
	Spiraea arborea	a
	S. japonica var. *Bumalda, Anthony Waterer*	d
	S. japonica var. *ruberrima*	e
	S. media	d
	S. Sargentiana	b
	Stephanandra incisa	d
	Stranvaesia Nussia	b
	S. salicifolia	d
	S. undulata	a
39–142.	*Deutzia scabra*	g
	Hydrangea petiolaris	e
	Philadelphus Wilsonii	g
39–141.	*Ribes aureum*	d
	R. lacustre	b
	R. speciosum	a
39–139.	*Escallonia edinensis*	d
35–130.	*Tarrietia Argyrodendron*	p
35–128.	*Elaeocarpus obovatus*	d
34–123.	*Hypericum hircinum*	b
	H. Hookerianum	a
	H. lysimachioides	d
	H. patulum var. *Forrestii*	a
33–118.	*Acmena floribunda*	v
	Callistemon citrinus var. *splendens*	l
	Eugenia rupestris	l
	Myrtus communis	C
	M. communis var. *tarentina*	A
32–114.	*Ochna multiflora*	e
32–108.	*Eurya ochnacea*	d
30–104.	*Begonia glaucophylla*	g
24–85.	*Hibbertia volubilis*	b
21–77.	*Epilobium hirsutum*	l
	Fuchsia magellanica var. *Riccartonii*	d
	F. magellanica var. *corallina*	r
	Oenothera fruticosa var. *Youngii*	g
	Oe. glauca var. *Fraseri*	n
21–75.	*Punica granatum* var. *flore pleno*	l
20–71.	*Impatiens biflora*	d
18–57.	*Polygonum baldschuanicum*	d
	P. polystachyum	d
	P. Sieboldii	d
	Rumex alpinus	b
13–42.	*Polygala vulgaris* var. *serpyllacea*	g
5–19.	*Berberis chitria*	d
	B. Lycium	d
	B. pruinosa	w
	B. Thunbergii	d
	B. Vernae	b
	B. virescens	d
	Mahonia aquifolium	d
4–15.	*Anemone nemorosa*	g
	Caltha palustris	g
	Cimicifuga racemosa	f
	Clematis Armandii	g
	C. Armandii var. *La Mortola*	g
	C. recta var. *purpurascens*	g
	Paeonia Darius	s
	P. Emodii	u
	P. Emodii var. *lobata*	s
	P. suffruticosa	u
	P. trolloides	t
	Thalictrum glaucum	j
3–10.	*Doryphora Sassafras*	e
1–2.	*Drimys Winteri*	d
1–1.	*Manglietia Hookeri*	u

Monocotyledons

Acorus Calamus variegatus	d	*Tradescentia bracteata*	g
Alpinia Sanderae	d	*T. virginiana*	g
Lilium regale	d		

Hybrids

Cistus corbariensis	a	*Phlox Coquelicot*	o
Diervilla hybrida	d	*Raphiolepis Delacourii*	e
Lychnis Arkwrightii	c	*Tritonia G. Davison*	n
Philadelphus purpureo-maculatus	g		

Bouganvillaea, Mrs Butt, young leaves contained a nitrogenous anthocyanin with behaviour similar to that found in the bracts [Price & Robinson, 1937].

a Cyanidin 3-monoside.
b Cyanidin monoside.
c Cyanidin 3-monoside + a little diglycoside.
d Cyanidin 3-PG.
e Cyanidin PG.
f Cyanidin 3-PG + a little diglycoside.
g Cyanidin 3-bioside.
h Cyanidin 3-bioside + cyanidin 3-PG.
i Cyanidin 3-bioside + cyanidin 3-monoside.
j Cyanidin 3:5-dimonoside.
k Acylated cyanidin 3:5-dimonoside.
l Cyanidin diglycoside.
m Acylated cyanidin diglycoside.
n Cyanidin diglycoside + cyanidin monoside.
o Cyanidin diglycoside + cyanidin PG.
p Cyanidin saccharide with very high distribution.
q Cyanidin 3-bioside + PG possibly containing some malvidin.
r Peonidin 3-bioside + some cyanidin 3-bioside.
s Peonidin 3:5-dimonoside + some peonidin monoside.
t Peonidin 3:5-dimonoside.
u Peonidin 3:5-dimonoside + some cyanidin 3:5-dimonoside.
v Malvidin 3:5-dimonoside.
w Malvidin PG.
x Malvidin 3-PG + trace Fe^{+} anthocyanin.
y Malvidin 3-PG + some cyanidin 3-PG.
z Malvidin 3-bioside + cyanidin 3-bioside.

A Delphinidin 3-PG.
B Delphinidin diglycoside + delphinidin PG.
C Malvidin 3-PG + delphinidin 3-PG.

(PG = pentoseglycoside.)

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